Renal segmental microvascular responses to ANG II in AT1A receptor null mice

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There are at least two major angiotensin receptors: AT1 and AT2. The AT1 receptor is thought to mediate most of the actions of ANG II on renal hemodynamic and tubular function, including afferent and efferent arteriolar vasoconstriction (3, 9, 31), modulation of tubuloglomerular feedback sensitivity (16), sodium and fluid reabsorption (18), and growth and differentiation (29). Two subtypes of the AT1 receptors, designated AT1A and AT1B, have been identified in the rat (7, 12, 13, 23) and mouse (24). The AT1A receptor is thought to be the predominant renal form. Terada et al. (28) reported localization of the AT1A mRNA in microdissected renal vascular segments (glomeruli, vasa recta bundle, and arcuate arteries) of the kidney by RT-PCR methods. Further studies identified AT1A and AT1B mRNAs in the same renal vascular structures, as well as the afferent arteriole (2, 7). Additionally, the AT1 receptor protein has been localized to the entire rat renal vasculature using immunohistochemical techniques and antibodies that recognize specifically the AT1A receptor (30) or both the AT1A and AT1B receptor subtypes (10, 17, 21, 30). The mRNA and protein expression profiles of the AT1 receptor subtypes have not been determined for the efferent arteriole. Furthermore, the contribution of the AT1A and AT1B receptors to the afferent and efferent arteriolar responses to ANG II have not been investigated.

The AT1A and AT1B receptors are pharmacologically indistinguishable from each other, and so it has not been possible to discriminate between the receptor subtype functions using pharmacological antagonists. The physiological effects of renal AT1A and AT1B receptor subtypes have yet to be elucidated, although the calcium signaling mechanisms of the AT1A and AT1B receptors appear to be identical in isolated cells (15, 32). The AT1A and AT1B receptor subtype localization, regulation, and function in various pre- and postglomerular microvascular segments may play an important part in the renal hemodynamic responses to ANG II in a variety of physiological and pathophysiological conditions.

Gene-targeted mice have proven to be a critical tool in defining the role of each AT1A and AT1B receptor subtype in vivo. AT1A−/− mice have reduced blood pressure, lack a systemic pressor response to exogenous ANG II, and exhibit mild renal structural abnormalities that include slight papillary hypoplasia and hyperplasia of renin-producing granular cells (11, 20, 26). Surprisingly, renal hemodynamics of wild-type (WT) and AT1A−/− mice are similar, such that glomerular filtration rate and renal plasma flow (6) and renal blood flow (22) do not differ between anesthetized WT and AT1A−/− mice; however, renal vascular resistance...
is lower in the AT1A−/− mice, paralleling the lower arterial pressure (22). The reduction in renal blood flow produced by ANG II in WT mice is similar to the reduction in renal blood flow produced by 10-fold higher doses of ANG II in AT1A−/− mice (22). However, the renal microvascular segment responsible for the ANG II responsiveness in AT1A−/− mice could not be determined in these studies. Additionally, AT1A−/− mice lack a tubuloglomerular feedback mechanism (25), which may result in an impaired renal autoregulatory responsiveness in these mice.

The present studies were conducted to test the hypotheses that AT1A−/− receptor-deficient mice display impaired renal afferent arteriolar autoregulatory responses and altered afferent and efferent arteriolar ANG II sensitivity. To directly assess the renal microvascular responses to changes in renal arterial perfusion pressure and ANG II, vessels were studied in an intact tubular environment (5) using the mouse in vitro blood-perfused juxtamedullary nephron technique, which is based on the rat in vitro blood-perfused juxtamedullary nephron technique originally developed by Casellas et al. (4). Kidneys were harvested from mice under pentobarbital sodium anesthesia (50 mg/kg ip). The renal artery was cannulated via the descending aorta under a dissecting microscope and immediately perfused with a Tyrode buffer containing 51 g/l bovine serum albumin (98–99% albumin, Sigma) and a mixture of L-amino acids at pH 7.4 as previously described in detail (8). The cannula system includes a 27-gauge blunted hypodermic needle for introduction into the renal artery, polyethylene (PE)-10 tubing for blood perfusion, and PE-10 tubing for the measurement of perfusion pressure. The tips of all of the tubing are in close proximity to each other. A section of liver was removed from each animal, immersed into liquid nitrogen, and stored at −70 °C for genotyping by Southern blot analysis as previously described (11). The kidney was placed in a perfusion chamber at room temperature for the dissection procedure, which included removal of the ventral third of the kidney, reflection of the papilla, cutting open of the renal veins, placement of 10.0 suture ties on the distal segments of the large arteries, and removal of the connective tissue and pelvic mucosa overlying the juxtamedullary cortical surface (Fig. 1A).

Blood was collected from a pentobarbital sodium anesthetized rat (50 mg/kg ip) through a carotid arterial cannula into a heparinized syringe. Blood was centrifuged, the buffy coat was discarded, and plasma and red blood cells (RBCs) were separated. RBCs were washed 2× in 0.9% NaCl, and the plasma was filtered (0.2, 5 μm). After completion of all microdissection procedures, the Tyrode perfusate was replaced with the reconstituted rat blood (hematocrit 33%). Microscopic examination of RBCs from the rat and mouse indicate that the diameters are similar (4–6 μm mouse; 5–7 μm rat). There was no indication that perfusion of the mouse kidney with rat blood causes any limitations in glomerular capillary blood flow or alterations in vascular responses. Renal arterial perfusion pressure was measured using a

**METHODS**

**Mouse in vitro blood-perfused juxtamedullary nephron technique.** Assessment of afferent and efferent arteriolar diameters was conducted in kidneys from 49 adult male and female WT (n = 14 females; n = 4 males), AT1A+/− (n = 4 males), and AT1A−/− (n = 22 females; n = 5 males) mice ranging from 3 to 7 mo of age that were bred in our colony at Duke University. Forty-nine adult male Sprague-Dawley rats were used as blood donors. Experiments were conducted using the mouse in vitro blood-perfused juxtamedullary nephron technique, which is based on the rat in vitro blood-perfused juxtamedullary nephron technique originally described (11). The kidney was placed in a perfusion chamber overlying the juxtamedullary cortical surface (Fig. 1A).

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**Fig. 1.** Mouse in vitro blood-perfused juxtamedullary nephron. A: photograph of the mouse kidney after the dissection procedure. The kidney is perfused via a 27-gauge needle. The pelvic mucosa is reflected up and held in place with pins revealing the underlying juxtamedullary nephrons. B: photograph of the mouse kidney on the stage of the videomicroscope. The chamber is warmed, and the kidney is superfused with solutions. C: digital image captured from the video monitor of a mouse blood-perfused afferent arteriole. The arrows demarcate the inside luminal borders. Bar = 15 μm.
P23XL transducer and a polygraph and was maintained at 100 mmHg by adjusting the regulator controlling the flow of the O₂-CO₂ mixture from the tank that pressurized the reservoir. The perfusion chamber was warmed, and the tissue surface was continuously superfused with an albumin-containing (10 g/l) Tyrode solution at 37°C. Agents were administered by addition to this bathing solution. The chamber was affixed to the stage of a Nikon Optiphot microscope equipped with dry objectives (×4, ×10, ×32) and a water-immersion objective (Zeiss, ×40/0.75 numerical aperture) (Fig. 1B). The tissue was transilluminated using a halogen lamp. The focused image of the vessel was transmitted via a high-resolution Newvicon camera (NC-67M, Dage-MTI) through a time-date generator (WJ-810, Panasonic) and an image-enhancing processor (MFJ-1452, MFJ Enterprises) and displayed on a monochromatic monitor (final magnification ×3,500). The video signal was recorded simultaneously on videotape for later analysis (SuperVHS VCR, Panasonic). Diameter measurements were obtained at a single site along the length of the selected vessel using an image-shearing monitor (Instrumentation for Physiology and Medicine, San Diego), and image-shearing diameters were measured using an image-shearing monitor (Instrumentation for Physiology and Medicine, San Diego). The image-shearing monitor diameter measurements were reproducible to within <0.1 µm. Afferent arterioles were studied near the glomerulus in the region sensitive to input from tubuloglomerular feedback. Afferent arterioles were studied near the glomerulus before peritubular capillary branching. Afferent arterioles (Fig. 1C) were measured at a site averaging 60 ± 2 µm from the glomerulus (n = 35). Afferent arteriolar total length averaged 280 ± 20 µm (n = 35). Afferent arterioles were measured at a site averaging 60 ± 3 µm from the glomerulus (n = 14). Experimental protocols were begun after a 10- to 15-min stabilization period. A single vessel was studied from each kidney, and only one kidney was studied from each animal.

**Afferent arteriolar autoregulatory responses.** Afferent arterioles were monitored in response to elevations in renal perfusion pressure in kidneys from female WT (n = 6), AT1A+/− (n = 2), and AT1A−/− (n = 6) mice. Afferent arterioles were measured during a 5-min control period at 100 mmHg. Renal perfusion pressure was increased in a stepwise fashion to 120, 140, and 160 mmHg as previously described for studies in the rat kidney (27). The pressures were maintained at each level for 3 min. Pressure was then returned to 100 mmHg for a 5-min recovery period. In a subset of mice, ANG II dose responses were determined after the recovery period, as described below.

**Afferent arteriolar ANG II responses.** Afferent arteriolar diameters in kidneys from male and female WT (n = 9 females; n = 2 males), AT1A+/− (n = 4 females) and AT1A−/− (n = 14 females; n = 3 males) mice were measured during superfusion with ANG II. After a 5-min control period or recovery from the change in perfusion pressure protocol, the kidneys were superfused sequentially with 0.1, 1.0, and 10 nM ANG II for a period of 5 min for each concentration. A recovery period of 5 min was then observed. Kidneys were then superfused for 5 min with an AT1 receptor blocker (1 µM candesartan), and the ANG II concentration-response relationship was repeated in the same vessel from WT (n = 5), AT1A+/− (n = 2), and AT1A−/− (n = 11) mice.

**Afferent arteriolar ANG II time control responses.** Afferent arteriolar diameters were measured in kidneys from female AT1A−/− mice (n = 3) in response to 0, 0.1, 1.0, 10, and 0 nM ANG II for a period of 5 min for each concentration. This protocol was repeated in the same vessels to demonstrate that the vasculature responds to a second application of ANG II.
Afferent and efferent arteriolar responses in AT1A−/− mice

Afferent arteriolar diameter responses to ANG II. Afferent arteriole diameter [μm (A); % of control (B)] responses to 0.1, 1, and 10 nM ANG II in kidneys from WT (○, n = 11) and AT1A−/− (●, n = 17) mice. ANG II produced a significant vasoconstriction in both groups. The afferent arteriolar vasoconstrictor response to 10 nM ANG II for AT1A−/− mice was significantly reduced compared with WT. Baseline diameters were not significantly different. *P < 0.05 vs. baseline. †P < 0.05 vs. WT.

Fig. 3. Afferent arteriolar diameter responses to elevations in renal perfusion pressure. Afferent arteriolar diameter [μm (A); % of control (B)] responses to stepwise increases in renal arterial perfusion pressure in kidneys from wild-type (WT; ○, n = 6) and AT1 receptor subtype-deficient (AT1A−/−; ●, n = 6) mice. Afferent arterioles from both groups responded to increases in perfusion pressure with a significant reduction in diameter. The magnitude of the afferent arteriolar vasoconstrictor responses were not significantly different between WT and AT1A−/− mice. *P < 0.05 vs. baseline.

Fig. 2. Afferent arteriolar diameter responses to elevations in renal perfusion pressure. Afferent arteriolar diameter [μm (A); % of control (B)] responses to stepwise increases in renal arterial perfusion pressure in kidneys from wild-type (WT; ○, n = 6) and AT1 receptor subtype-deficient (AT1A−/−; ●, n = 6) mice. Afferent arterioles from both groups responded to increases in perfusion pressure with a significant reduction in diameter. The magnitude of the afferent arteriolar vasoconstrictor responses were not significantly different between WT and AT1A−/− mice. *P < 0.05 vs. baseline.

Afferent arteriolar diameter responses to ANG II. Afferent arteriole diameter [μm (A); % of control (B)] responses to 0.1, 1, and 10 nM ANG II in kidneys from WT (○, n = 11) and AT1A−/− (●, n = 17) mice. ANG II produced a significant vasoconstriction in both groups. The afferent arteriolar vasoconstrictor response to 10 nM ANG II for AT1A−/− mice was significantly reduced compared with WT. Baseline diameters were not significantly different. *P < 0.05 vs. baseline. †P < 0.05 vs. WT.

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from $\text{AT}_{1A}^{-/-}$ were not different in those from WT or $\text{AT}_{1A}^{-/-}$ mice.

At the completion of application of ANG II, vessels were exposed to the control superfusion for a recovery period. The AT$_1$ receptor antagonist candesartan (1 $\mu$M) was applied to the surface of the kidney for a period of 5 min. Diameters were not altered by candesartan alone in WT ($n = 5$), $\text{AT}_{1A}^{+/+}$ ($n = 2$), or $\text{AT}_{1A}^{-/-}$ ($n = 11$) vessels, averaging $100 \pm 1$, 105, and $100 \pm 1\%$ of control, respectively. Moreover, the AT$_1$ receptor antagonist completely blocked the afferent arteriolar responses to all doses of ANG II in all groups, demonstrating that the afferent arteriole vasoconstrictor responses are mediated by the AT$_1$ receptor subtypes.

**Afferent arteriolar ANG II time control responses.** This series of experiments was performed to demonstrate that afferent arterioles respond to repeated application of increasing concentrations of ANG II. Afferent arteriolar diameter in kidneys from $\text{AT}_{1A}^{-/-}$ mice ($n = 3$) averaged $17.7 \pm 0.1$ $\mu$m at baseline. Application of 0.1, 1, and 10 nM ANG II produced graded reductions in afferent arteriolar diameter of $6 \pm 1$, $15 \pm 1$, and $23 \pm 1\%$ of control levels, respectively (Fig. 4). On the second application of 0.1, 1, and 10 nM ANG II, vessel diameters decreased $6 \pm 1$, $14 \pm 1$, and $19 \pm 2\%$ of control levels, respectively. The afferent arteriolar responses to the first and second applications of ANG II did not differ significantly. Afferent arteriolar diameters were not significantly different between baseline and the two recovery periods. These data provide evidence that mouse juxtamedullary afferent arterioles do not display tachyphalaxis to ANG II at the concentrations used and respond actively to a repeat application of the peptide under these experimental conditions.

**Efferent arteriolar ANG II responses.** Efferent arteriolar baseline diameters in kidneys from $\text{AT}_{1A}^{-/-}$ mice were significantly larger than in those from WT mice, averaging $19.6 \pm 0.7$ and $17.0 \pm 0.3$ $\mu$m, respectively. Efferent arterioles of WT mice vasoconstricted in response to 0.1, 1, and 10 nM ANG II by $6 \pm 1$, $11 \pm 1$, and $21 \pm 5\%$ of control (Fig. 5). However, efferent arterioles from $\text{AT}_{1A}^{-/-}$ mice did not respond to ANG II. The AT$_1$-receptor antagonist candesartan alone did not alter efferent arteriolar diameter (100% of control) of WT ($n = 6$) or $\text{AT}_{1A}^{-/-}$ ($n = 6$) mice. As shown in Fig. 5, blockade of the AT$_1$ receptor with candesartan completely prevented the efferent arteriolar vasoconstrictor responses to ANG II in kidneys from WT mice.

**DISCUSSION**

The AT$_1$ receptor is primarily responsible for the vascular and tubular actions of the renal renin-angiotensin system. There are two unique AT$_1$ receptor subtypes in rodents, AT$_{1A}$ and AT$_{1B}$, which cannot be distinguished using pharmacological antagonists. Accordingly, it has not been possible to discriminate between renal microvascular AT$_{1A}$ and AT$_{1B}$ receptor subtype function. It is known that AT$_{1A}$ and AT$_{1B}$ receptor mRNAs are expressed on the afferent arteriole (2, 17). However, there is no information on the localization of AT$_{1B}$ receptor mRNA or protein on the efferent arteriole. Therefore, the purpose of the present study was to determine the functional contribution of the AT$_{1A}$ and AT$_{1B}$ receptors to the renal microvascular responses to changes in renal perfusion pressure and the segment-specific vasoconstrictor actions of ANG II.

The requirement for AT$_{1A}$ receptors for afferent arteriolar vascular control mechanisms with regard to elevations in renal arterial perfusion pressure were
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examinined in the present study using gene-targeted mice. Although AT1A receptor-deficient mice have been shown to lack a tubuloglomerular feedback mechanism (25), the present study revealed that increases in renal perfusion pressure evoke indistinguishable afferent arteriolar vasoconstriction only in vessels from WT mice. Baseline diameters were significantly larger in AT1A−/− compared with WT vessels.

arteriolar blood flow was efficiently autoregulated over the same pressure range (27). Thus afferent arteriolar autoregulatory responsiveness in the isolated perfused mouse kidney is similar to that previously observed in the rat and does not appear to be altered by the loss of AT1A receptor function. The maintenance of renal autoregulatory responsiveness in AT1A−/− mice suggests a prominence of the myogenic mechanism in these animals. Alternatively, tubuloglomerular feedback responses may be active in the deep, juxtamedullary nephron population of AT1A−/− mice and may reflect mediation by the AT1B receptor.

The relative contributions of AT1A and AT1B receptors to afferent arteriolar resting tone were evaluated in the present study. Afferent arteriolar diameters were not significantly different under baseline conditions (100 mmHg renal perfusion pressure, superfusion with vehicle solution) in kidneys from WT and AT1A−/− mice. The lack of a between-group difference in baseline diameter indicates no effect of loss of AT1A receptors on basal afferent arteriolar tone. In addition, basal afferent arteriolar diameters were not altered after AT1 receptor blockade by candesartan in kidneys from WT or AT1A−/− mice. This suggests that there is little influence of ANG II on basal afferent arteriolar tone under the conditions of the isolated perfused mouse kidney preparation.

The relative contributions of AT1A and AT1B receptors to afferent arteriolar responses to ANG II were determined in kidneys from WT, AT1A+/+ and AT1A−/− mice. The afferent arteriolar diameter responses to ANG II in WT mice were similar to those previously reported in the rats pretreated with enalaprilat (3, 9). Significant reductions in afferent arteriolar diameters were observed over the concentration range of 0.1–10 nM in kidneys from WT, AT1A+/+, and AT1A−/− mice. We attribute the ANG II vasoconstrictor responses in afferent arterioles from AT1A−/− mice to be mediated by the AT1B receptor subtype. Surprisingly, the magnitude of the afferent arteriolar responses in the three groups of mice was similar at the 0.1 and 1 nM ANG II doses. This was unexpected based on previous studies, which demonstrated a negligible pressor response to bolus systemic ANG II (11) and diminished renal blood flow response to bolus intrarenal administration of ANG II (22) in kidneys from AT1A−/− mice compared with controls. However, it has been shown that AT1A−/− mice have significantly enhanced renal renin mRNA expression (19) and elevated plasma ANG II levels (6). Elevated circulating ANG II may have contributed to the lack of ANG II response in kidneys from AT1A−/− mice in the above studies. Endogenous ANG II may occupy the AT1B receptors and limit accessibility of exogenously administered ANG II. In fact, after administration of an angiotensin-converting enzyme inhibitor, both systemic pressor and renal blood flow responses to ANG II were enhanced (6, 19). Therefore, suppression of the endogenous production of ANG II may be necessary to reveal the function of the AT1B receptor in the absence of the AT1A receptor. It is possible that circulating ANG II levels are low.

Fig. 5. Efferent arteriolar diameter responses to ANG II. Efferent arteriolar diameter (μm A; % of control B) responses to 0.1, 1.0, and 10 nM ANG II in kidneys from WT (○, n = 6) and AT1A−/− (●, n = 6) mice. ANG II produced a significant efferent arteriolar constriction only in vessels from WT mice. Baseline diameters were significantly larger in AT1A−/− compared with WT vessels.*P < 0.05 vs. baseline. †P < 0.05 vs. WT.
using the mouse juxtamedullary nephron technique, in which the kidney is perfused with blood obtained from a donor rat and, therefore, afferent arteriolar responses in kidneys from AT1A−/− mice are revealed.

In contrast to the afferent arteriolar vasoconstrictor responses to low-dose ANG II, afferent arteriolar diameter responses to the high dose of ANG II, 10 nM, were significantly different in kidneys from WT and AT1A−/− mice. Afferent arteriolar diameter responses for the AT1A−/− mice were only 60% of the magnitude of the response for WT mice. The difference in the magnitude of the response may be due to the maximal vasoconstrictor contribution of the AT1B receptors. The vasoconstrictor responses to ANG II were completely inhibited by the AT1 receptor blocker candesartan. This drug blocks both the AT1A and AT1B receptor subtypes, similar to the properties of losartan (15). Therefore, the vasoconstrictor effects of ANG II on the afferent arteriole are mediated by the AT1 receptor for both WT and AT1A−/− mice. There was no evidence of AT2 receptor-mediated vasodilation in the presence of AT1 receptor blockade and ANG II in the present study. We conclude that for WT mice, afferent arteriolar responses are mediated by both the AT1A and AT1B receptors, whereas for AT1A−/− mice, this effect is mediated exclusively by the AT1B receptors. In addition, in the absence of AT1A receptors, 10 nM ANG II evokes an attenuated, candesartan-sensitive, afferent arteriolar constriction in kidneys from AT1A−/− mice, implicating activation of AT1B receptors. It is not known at the present time whether AT2 and/or AT1B receptor protein expression is altered in afferent arterioles of AT1A−/− mice.

The relative contributions of AT1A and AT1B receptors to efferent arteriolar responses to ANG II were determined in kidneys from WT and AT1A−/− mice. Efferent arterioles of WT mice responded in a dose-dependent manner to ANG II, similar to juxtamedullary efferent arterioles of the rat (3, 9). However, efferent arterioles of AT1A−/− mice did not respond to ANG II. These data suggest that AT1A receptors are primarily responsible for ANG II-induced efferent arteriolar vasoconstriction. The lack of ANG II responses in efferent arterioles of AT1A−/− mice suggests that AT1B receptors are not functionally expressed on the efferent arteriole.

In contrast to the similarities in the afferent arteriolar resting diameters of WT and AT1A−/− mice, efferent arteriolar diameters of AT1A−/− mice were significantly larger than for WT mice. Such increased efferent arteriolar diameter combined with an increased glomerular ultrafiltration coefficient, resulting from reduced ANG II-dependent activation of AT1A receptors, may contribute to the maintenance of renal plasma flow and glomerular filtration rate in the normal range in hypotensive AT1A−/− mice (6). However, there may be limitations to our ability to extrapolate our data obtained from in vitro studies to in vivo setting. It is not likely that the larger resting efferent arteriolar diameter in kidneys from AT1A−/− mice is a result of the direct loss of the effects of endogenous ANG II on the AT1A receptor because resting diameter was not influenced by candesartan. At this time, we can only speculate on the potential interaction of ANG II-induced vasoconstriction and other vasodilatory mechanisms at the site of the efferent arteriole. The larger resting efferent arteriolar diameter of AT1A−/− mice may reflect a lack of compensation by the vasoconstrictor properties of the AT1A receptor. It has been shown that AT1A−/− mice have increased expression and activity of neuronal nitric oxide synthase (14). Because nitric oxide derived from neuronal nitric oxide synthase localized in the macula densa cells and efferent arterioles (1) has been shown to play an important role in renal hemodynamics, it is possible that nitric oxide has profound effects on the resting tone of the efferent arteriole lacking AT1 receptors.

In conclusion, afferent arteriolar autoregulatory capability is not affected by the absence of AT1A receptors. This study in AT1A−/− mice provides functional evidence of distinct distribution patterns for AT1 receptor subtypes within the renal microvasculature. We conclude that afferent arteriolar vasoconstrictor responses to ANG II are mediated by AT1A and AT1B receptors, whereas efferent arteriole vasoconstrictor responses to ANG II are mediated by AT1A receptors in the mouse kidney.

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