Sex-Specific Influence of Angiotensin Type 2 Receptor Stimulation on Renal Function

A Novel Therapeutic Target for Hypertension

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Abstract—The renin-angiotensin system is a powerful regulator of arterial pressure and body fluid volume. Increasing evidence suggests that the angiotensin type 2 receptor (AT_2R), which mediates the vasodilatory and natriuretic actions of angiotensin peptides, is enhanced in females and may, therefore, represent an innovative therapeutic target. We investigated the therapeutic potential of direct AT_2R stimulation on renal function in 11- to 12-week–old anesthetized male and female Sprague-Dawley rats. Renal blood flow was examined in response to a graded infusion of the highly selective, nonpeptide AT_2R agonist, compound 21 (100, 200, and 300 ng/kg per minute), in the presence and absence of AT_2R blockade (PD123319; 1 mg/kg per hour). Direct AT_2R stimulation significantly increased renal blood flow in both males and females, without influencing arterial pressure. This was dose dependent in females only and occurred to a greater extent in females at the highest dose of compound 21 administered (males: 13.1±2.4% versus females: 23.0±3.2% change in renal blood flow at 300 ng/kg per minute versus baseline; $P<0.01$). In addition, AT_2R stimulation significantly increased sodium and water excretion to a similar extent in males and females ($P_{Group}=0.05$ and 0.005). However, there was no significant change in glomerular filtration rate in either sex, suggesting that altered tubular function may be responsible for AT_2R-induced natriuresis rather than hemodynamic effects. Taken together, this study provides evidence that direct AT_2R stimulation produces vasodilatory and natriuretic effects in the male and female kidney. The AT_2R may, therefore, represent a valuable therapeutic target for the treatment of renal and cardiovascular diseases in both men and women. **(*Hypertension*. 2012;59[part 2]:409-414.)**

Key Words: angiotensin type 2 receptor ■ compound 21 ■ sex differences ■ hypertension ■ natriuresis ■ renal blood flow

Before menopause, women are protected from hypertension and cardiovascular disease relative to men. However, this protection weakens after menopause, and ultimately the prevalence of hypertension in women exceeds that of men.[1] Alarmingly, sex-related differences have also been reported in the efficacy of current cardiovascular therapies,[2,3] with poorer treatment outcomes commonly reported in women.[3] The development of sex-specific approaches for the treatment of hypertension and cardiovascular disease is, therefore, of utmost importance.

It is well established that the kidney plays a central role in arterial pressure control. The regulation of sodium excretion by the kidney is critical to the long-term regulation of arterial pressure given its influence on body fluid volume homeostasis.[4] In response to a rise in arterial pressure, sodium and water excretion is increased to reduce extracellular fluid volume and to restore arterial pressure to normal. Abnormal renal excretory function is, therefore, recognized as a key contributor to the development of hypertension.[5]

The renin-angiotensin system (RAS) plays a pivotal role in the regulation of arterial pressure by the kidney because of its influence on renal excretory function and vascular tone.[4] The majority of the classically recognized actions of angiotensin II (Ang II) are mediated by the angiotensin type 1 receptor (AT_1R), including vasoconstriction, sodium reabsorption, and cell proliferation.[6] However, in recent years, a depressor axis of the RAS has been discovered. In addition to Ang II, other biologically active peptides derived from angiotensin I and Ang II directly oppose the classic pressor actions of Ang II mediated by the AT_1R, by promoting vasodilation and natriuresis via their interaction with the angiotensin type 2 receptor (AT_2R).[7]

Accumulating evidence suggests that the vasodepressor RAS pathways are enhanced in females. We, and others, have identified major sex differences in the expression level of various RAS components,[8,9] together with differences in the male and female response to RAS activation and inhibition.[3,8,10,11] Of particular interest, increased AT_2R expression has been identified in the kidney and vasculature of female

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mice and rats compared with their male counterparts.[8,12] In both male and female rats, we recently identified a significant role for the AT_2R in the control of renal function. We reported that the AT_2R modulates sodium excretion in both males and females to a similar extent. In addition, in females, we identified a sex-specific role for the AT_2R in the renal vasculature, providing protection against the vasoconstrictor effects of Ang II.[13] Thus, increased renal expression of the AT_2R in females may underlie their cardiovascular protection, and, as such, therapeutic stimulation of the AT_2R could counterbalance the pressor actions of the RAS and may be a suitable target for future cardiovascular treatments.

In this study we aimed to further define the sex-specific role of the AT_2R in the regulation of arterial pressure via the modulation of renal hemodynamics and excretory function. We examined the influence of direct AT_2R stimulation on renal function in male and female rats, using the highly specific nonpeptide AT_2R agonist, compound 21. We hypothesized that direct AT_2R stimulation would promote sodium excretion and renal vasodilation to a greater extent in females.

Methods

Animals

Ten-week–old male and female Sprague-Dawley rats were obtained from the Animal Resources Centre (Perth, Western Australia, Australia) and were fed a sodium-controlled diet (0.25% sodium chloride; Specialty Feeds, Glen Forrest, Western Australia, Australia) and water ad libitum. The rats were individually housed with a 12-hour light/dark cycle at a temperature of 21°C and were allowed 1 week to acclimatize. Experiments were approved by the Monash University, School of Biomedical Sciences Animal Ethics Committee, and were performed in accordance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes.

Surgical Procedure

The rats were prepared as described in detail previously.[13] Briefly, rats were anesthetized with Inactin (thiobutabarbital; 150 mg/kg; Sigma Aldrich, St Louis, MO), and a tracheostomy was performed to facilitate breathing. The left jugular vein and carotid artery were catheterized for IV infusion of 2% BSA (Sigma Aldrich) and tritiated inulin ([^{3}H]inulin; Sigma Aldrich) and mean arterial pressure (MAP) measurement, respectively. Next, the left kidney was exposed and denervated and the left ureter was catheterized for urine collection. Finally, a transit-time ultrasound flow probe (0.7VB; Transonic Systems, Ithaca, NY) was placed around the renal artery for renal blood flow (RBF) measurement.

Experimental Procedures

After surgery, PD123319 (1 mg/kg bolus plus 1 mg/kg per hour; Sigma Aldrich) or vehicle (0.9% saline; 1 mL bolus plus 1 mL/h) was administered intravenously for the duration of the experiment. After 30 minutes, intravenous infusion of constant vehicle or graded compound 21 (0, 100, 200, and 300 ng/kg per minute) commenced. At each dose, after a 10-minute equilibration period, RBF measurements were obtained for 5 minutes. Urine produced by the left kidney was collected during the baseline and 300 ng/kg per minute collection periods, and corresponding arterial blood samples were taken from the left carotid artery at the period end. Urinary sodium concentrations were measured as described previously,[13] and fractional sodium excretion (FENa) was calculated. Glomerular filtration rate was estimated based on [^{3}H]inulin clearance. At the end of the experiment the left kidney was excised and weighed. The stage of the estrus cycle of each female rat was also identified by a vaginal smear, and uterine weight was measured. Compound 21 was provided by U.M.S. and T.U. (Charité-Universitätsmedizin Berlin, Berlin, Germany).

Statistical Analysis

Data are expressed as mean±SEM. To compare differences in physiological parameters and baseline values, data were analyzed using an ANOVA with Tukey post hoc tests. MAP, RBF, and renal vascular resistance (RVR) data were analyzed using repeated-measures ANOVA with Bonferroni post hoc tests. To compare differences in percentage changes in variables at 300 ng/kg per minute compound 21 from baseline between treatment groups, data were analyzed using a 2-way ANOVA. P values <0.05 were considered statistically significant.

Results

Physiological Parameters

Both body weight (BW) and left kidney wet weight (KW) were significantly greater in the male vehicle-treated (BW: 427±9 g; left KW: 1.40±0.04 g), compound 21-treated (BW: 421.0±10.9 g; left KW: 1.50±0.05 g), and compound 21+PD123319-treated (BW: 438±12 g; left KW: 1.50±0.06 g) groups as compared with the female vehicle-treated (BW: 263±7 g; left KW: 0.92±0.03 g), compound 21-treated (BW: 271±9 g; left KW: 0.98±0.03 g), and compound 21+PD123319-treated groups (BW: 254±4 g; left KW: 0.88±0.02 g; $P_{Sex}<0.0001$). Uterine weight did not differ significantly between the female vehicle-treated (0.60±0.05 g), compound 21-treated (0.55±0.04 g), and compound 21+PD123319-treated (0.52±0.04 g) groups. Furthermore, each female group consisted of both estrus and anestrus rats, in equal proportion.

Influence of Direct AT_2R Stimulation on Renal Hemodynamics

There were no significant differences in basal MAP between the male and female treatment groups (Table). In response to the vehicle, graded compound 21 infusion, or graded compound 21 infusion combined with PD123319, MAP remained near baseline levels throughout the duration of the experiment (Figure 1A and 1D). This response was similar between the male and female treatment groups; however, there was a small but significant (≈4 mm Hg) rise in MAP in the male compound 21-treated group across the time course of the experiment, as compared with the male compound 21+PD123319-treated group ($P_{Group}=0.02$).

In males, baseline RBF was similar among the 3 treatment groups (Table). In response to vehicle infusion, there was no significant change in RBF from the baseline level. In contrast, RBF increased significantly in response to compound 21 infusion ($P_{Group}=0.0009$; Figure 1B). At 300 ng/kg per minute of compound 21, RBF was increased by 13.1±2.4% as compared with baseline ($P_{300}>0.001$). This renal vasodilatory response to compound 21 in males was also evidenced by reduced RVR, as compared with the male vehicle-treated group ($P_{Group}=0.008$; Figure 1C). However, although a significant increase in RBF was observed after compound 21 infusion in males, this response was not dose dependent ($P_{Dose}>0.05$). This effect was completely abolished in the presence of AT_2R blockade with PD123319 ($P_{Group}=0.006$; Figure 1B).

Likewise in females, baseline RBF did not differ significantly between the treatment groups (Table). In response to vehicle infusion, RBF remained near baseline levels but

Table. Baseline Levels of Mean Arterial Pressure, Renal Hemodynamics, Urine Flow, and Urinary Sodium Excretion

Group	MAP, mm Hg	RBF, mL/min/g	GFR, mL/min/g	UF, μl/min/g	$U_{Na+}V$, μmol/min/g
Male vehicle	119±5	3.8±0.2	1.3±0.1	6.3±1.5	0.3±0.1
Male compound 21	117±3.0	4.0±0.1	1.5±0.1	5.2±0.5	0.2±0.04
Male compound 21+PD123319	122±4	3.8±0.2	1.3±0.2	7.0±3.9	0.6±0.5
Female vehicle	116±3	3.3±0.2	1.4±0.05	8.5±1.6	0.6±0.2
Female compound 21	118±5	3.5±0.2	1.7±0.1	8.9±2.6	0.5±0.2
Female compound 21+PD123319	112±3	3.8±0.3	1.4±0.2	8.6±1.3	0.5±0.2

MAP indicates mean arterial pressure; RBF, renal blood flow; GFR, glomerular filtration rate; UF, urine flow; and $U_{Na+}V$, urinary sodium excretion. Data are presented as mean±SEM and were analyzed using an ANOVA with Tukey post hoc tests. n=6 to 10 per group. All of the RBF, GFR, UF, and $U_{Na+}V$ values are expressed per gram of left wet kidney weight.

increased significantly in response to compound 21 treatment (P_{Group}<0.0001; Figure 1E). RBF increased by 23.0±3.2% from baseline in the presence of 300 ng/kg per minute of compound 21 (P_{300}>0.001). However, unlike in males, the renal vasodilatory response to compound 21 observed in females was dose dependent (P_{Dose}<0.0001). This in turn resulted in a dose-dependent reduction in RVR as compared with vehicle treatment (Figure 1F). Each of these effects in females was abolished by coinfusion of PD123319 (P_{Group}=0.0006 for RBF and 0.001 for RVR; Figure 1E and 1F). Notably, there was a trend for the magnitude of the effect of compound 21 on RBF to be greater in females as compared with males (P_{Sex}=0.059). The reduction in RVR was also significantly greater in the female compound 21-treated versus the male compound 21-treated group (P_{Sex}=0.04). These sex differences were particularly evident at the

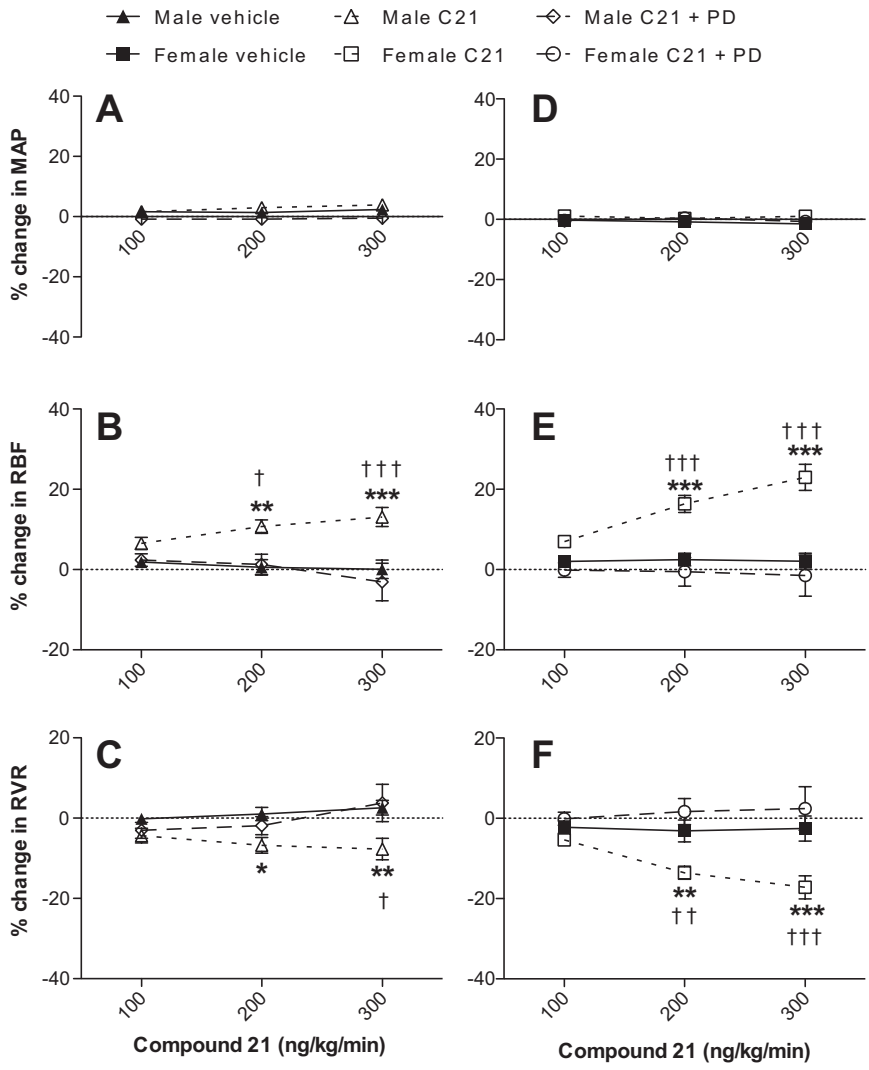

Figure 1. Percentage changes from baseline for mean arterial pressure (MAP), renal blood flow (RBF), and renal vascular resistance (RVR) in male (**A** through **C**) and female (**D** through **F**) rats in response to 100 to 300 ng/kg per minute of compound 21. Data are presented as mean±SEM and were analyzed using repeated-measures ANOVA with Bonferroni post hoc tests. *P<0.05, **P<0.01, ***P<0.001 vs vehicle-treated rats; †P<0.05, ††P<0.01, †††P<0.001 vs compound 21+PD123319-treated rats. n=6 to 10 per group. All of the RBF and RVR values are expressed per gram of left wet kidney weight.

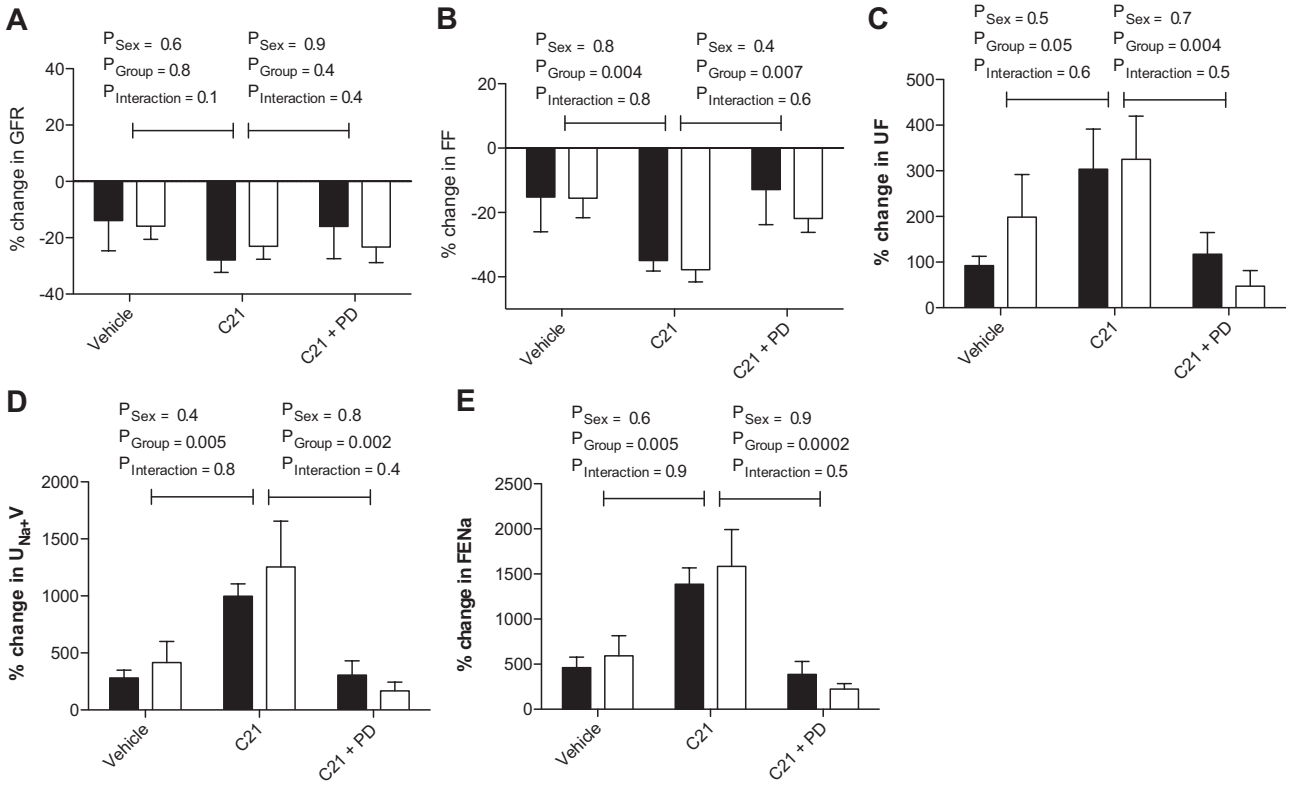

Figure 2. Percentage changes from baseline for (**A**) glomerular filtration rate (GFR), (**B**) filtration fraction (FF), (**C**) urine flow (UF), (**D**) sodium excretion ($U_{Na+}V$), and (**E**) fractional sodium excretion (FENa) in male (■) and female (□) rats in response to 300 ng/kg per minute of compound 21. Data are presented as mean±SEM and were analyzed using a 2-way ANOVA using the factors sex (P_{Sex}), group (P_{Group}), and the interaction between sex and group ($P_{Interaction}$). n=5 to 7 per group. All of the values are expressed per gram of left wet kidney weight.

highest dose of compound 21 administered ($P_{300}<0.01$ for RBF and RVR).

Influence of Direct AT_2R Stimulation on Renal Excretory Function

Renal excretory function was also examined during the baseline and 300 ng/kg per minute of compound 21 experimental periods in a subset of animals. Baseline glomerular filtration rate (GFR) was similar between the male and female treatment groups (Table). In response to vehicle treatment, we observed a time-dependent reduction in GFR. However, in response to 300 ng/kg per minute of compound 21, a similar reduction in GFR was observed in both the male and female rats, which did not differ significantly from that observed in the vehicle-treated groups ($P_{Group}>0.05$; Figure 2A). Subsequently, the observed changes in RBF and GFR resulted in a greater reduction in filtration fraction in response to compound 21 as compared with vehicle treatment ($P_{Group}=0.004$; Figure 2B). This was not a sex-dependent effect and was ameliorated by coinfusion of PD123319.

In terms of urine flow (UF) and urinary sodium excretion ($U_{Na+}V$), baseline levels did not differ significantly between the treatment groups of either sex (Table). In both males and females, a time-dependent increase in UF, $U_{Na+}V$, and FENa was observed in response to vehicle treatment (Figure 2C through 2E). UF, $U_{Na+}V$, and FENa were also increased in response to infusion of 300 ng/kg per minute of compound 21 in males and females. However, this occurred to a significantly greater extent than was observed in the vehicle-treated groups ($P_{Group}=0.05$ for UF and 0.005 for $U_{Na+}V$ and FENa). The magnitude of the UF, $U_{Na+}V$, and FENa responses to compound 21 was similar between the sexes ($P_{Sex*Group}>0.05$) and was abolished in both males and females by coinfusion of compound 21 and PD123319 (Figure 2C through 2E).

Discussion

The major finding observed in this study was that direct AT_2R stimulation using the AT_2R agonist, compound 21, induced renal vasodilation and natriuretic effects in both normotensive male and female rats. The renal vasodilatory effects of compound 21 were dose dependent in females only and were observed to a greater extent in females than males at higher concentrations of compound 21. These observations support the conclusion that direct AT_2R stimulation with compound 21 has a sex-specific influence on renal function. In addition, compound 21 administration caused similar increases in sodium and water excretion in males and females. To the best of our knowledge, this is the first study that has investigated the sex-specific influence of direct AT_2R stimulation on renal hemodynamics and excretory function.

In the past, investigations into the effects of AT_2R stimulation have been limited because of the restricted availability of metabolically stable AT_2R agonists.[14] Recently, the highly specific nonpeptide AT_2R agonist, compound 21, has become available. It is estimated that, because of its nonpeptide nature, compound 21 has an approximate bioavailability of 20% to 30%

and a 4-hour half-life in rats.[15] Moreover, compound 21 exhibits a similar biological response to Ang II and the commonly used peptide AT$_2$R agonist, CGP42112, but with minimal affinity for the AT$_1$R.[15] It is, therefore, widely becoming recognized as an ideal research tool suitable for direct investigations into the effects of AT$_2$R stimulation.[16]

In the present study, the dose selection of compound 21 was based on our observations from previous studies where we assessed the dose-dependent vascular effects of compound 21 in conscious spontaneously hypertensive rats, across a wide dose range.[14] In the presence of AT$_1$R blockade, acute administration of compound 21 lowered arterial pressure at doses ranging from 50 to 300 ng/kg per minute. This effect was completely abolished by coinfusion of PD123319. This selectivity for the AT$_2$R, however, appeared to be lost at higher doses of compound 21 tested (1000 ng/kg per minute), as indicated by increasing arterial pressure. Wan et al[15] also observed a similar bell-shaped dose-response relationship in anesthetized spontaneously hypertensive rats.

In the current study, intravenous infusion of compound 21 had a marked influence on renal hemodynamic function. We observed a significant increase in RBF in both male and female rats. This renal vasodilatory response was also reflected by a significant reduction in RVR. In combination with AT$_2$R blockade using PD123319, these effects were abolished, thereby supporting the role of the AT$_2$R in these responses. As we have discussed in detail previously, blockade of the AT$_1$R is generally required to inhibit circulating endogenous Ang II from exerting tonic AT$_1$R-mediated vasoconstriction for AT$_2$R-mediated vasodilation to be manifest.[17] Remarkably, the increased renal vasodilatory response to compound 21 in the present study was observed in the absence of any AT$_1$R blockade, demonstrating the ability of compound 21 to directly modulate renal function.

At higher concentrations of the AT$_2$R agonist, the renal vasodilatory response was significantly different between the sexes. At the highest dose of compound 21 administered, females demonstrated a more pronounced increase in RBF than males. In fact, in males the RBF response to graded infusion of compound 21 was not dose dependent. This may indicate that, in males, the peak receptor saturation response to compound 21 was reached at a lower dose than in females. In support of this notion, a lower abundance of the AT$_2$R has been identified previously in the male versus female renal vasculature. Studies examining angiotensin receptor expression in rodents have reported AT$_2$R expression in the glomeruli, afferent arterioles, arcuate arteries, and medullary descending vasa recta,[12,18,19] with evidence of greater AT$_2$R expression in the female vasculature.[18]

The sex-specific response to AT$_2$R stimulation is also consistent with our findings reported recently in normotensive rats that the AT$_2$R plays a more significant role in the female versus male renal vasculature.[13] In response to exogenous administration of Ang II, we previously observed dose-dependent decreases in RBF in both males and females. In the presence of AT$_2$R blockade with PD123319, RBF was further reduced in females alone, indicating a protective role of the AT$_2$R in females against the renal vasoconstrictor effects of Ang II.[13] Although we did not previously observe a significant role for the AT$_2$R in the male renal vasculature, it should be recognized that examination of the effects of AT$_2$R blockade on renal function alone does not necessarily reflect the potential for AT$_2$R activation to directly modulate renal function. As we have reviewed in detail previously, whereas evidence suggests the AT$_2$R may be constitutively active, the permanent endogenous activity of the AT$_2$R is much lower than its activity during pharmacological stimulation.[20] Indeed, our present findings suggest that AT$_2$R stimulation may provide renoprotective effects in both sexes. However, it must be considered when interpreting these results that these experiments were performed in denervated kidneys to avoid the potentially confounding influence of changes in renal sympathetic activity on renal function.

In the current study, we also observed significant increases in sodium and water excretion in response to AT$_2$R stimulation with compound 21 in both males and females. Recently, we reported that the AT$_2$R modulates sodium excretion in both sexes. In male and female normotensive rats, blockade of the AT$_2$R was shown to blunt the pressure-natriuresis relationship, suggesting that the AT$_2$R enhances the natriuretic capacity of the kidney.[13] In the present study we provided further evidence that supports these findings. AT$_2$R stimulation by compound 21 significantly increased sodium and water excretion in both male and female rats, an effect abolished by concomitant infusion of compound 21 and PD123319. Moreover, the increase in $U_{Na+}V$ and UF was similar in the male and female AT$_2$R agonist-treated rats. Thus, AT$_2$R stimulation appears to have similar effects on sodium excretion between the sexes.

As we have discussed in detail previously,[13] the natriuretic action of the AT$_2$R has been associated with NO-mediated changes in renal hemodynamics and/or tubular sodium handling. It is well documented that AT$_2$R activation in the vasculature stimulates bradykinin and NO and cGMP production, resulting in vasodilation.[7] This vasodilatory cascade has also been linked to the inhibition of sodium transport in the renal tubules.[21–23] Indeed, in addition to its localization in the renal vasculature mentioned previously, AT$_2$R expression has also been identified in the renal tubules, particularly within proximal tubular cells.[19,24] Interestingly, despite the change in renal excretory function in response to compound 21 in males and females, we did not see any significant change in GFR at the highest concentration of compound 21 in either sex. Given the major increase in RBF, a significant reduction in filtration fraction was, therefore, observed. The most likely explanation for this reduction in filtration fraction is that compound 21 has similar dilatory effects on both the preglomerular and postglomerular arterioles. This would result in increases in RBF, whereas GFR remains unchanged. Alternatively, we cannot rule out the possibility that AT$_2$R stimulation has a marked influence on the glomerular capillary ultrafiltration coefficient. Previous studies by Suzuki et al[25] have identified a role for the AT$_2$R in the maintenance of glomerular barrier function attributed to its presence on podocytes. They showed that AT$_2$R-mediated action enhances the expression of slit diaphragm-associated molecules. It is, therefore, plausible that AT$_2$R stimulation in our study resulted in relaxation of podocytes, reducing the surface area available for filtration. This reduction in ultrafiltration coefficient could counterbalance any increase in glomerular capillary pressure induced by AT$_2$R stimulation, leading to the no change in GFR that we

observed. Investigations into the exact localization of the angiotensin receptors in the glomerular vasculature could provide further insight into these observations. Nonetheless, a change in GFR does not appear to be a factor facilitating AT_2R-induced natriuresis. Rather, these collective observations suggest that the renal tubule is the major site of action, and the increased excretion of sodium in response to AT_2R stimulation is attributed to an inhibition of tubular sodium reabsorption, independent of AT_2R-mediated changes in renal hemodynamics. This is further supported by the increase in fractional excretion of sodium in both the male and female compound 21–treated rats.

In terms of future studies, it would be of interest to investigate the influence of direct pharmacological AT_2R stimulation on renal hemodynamic and excretory function in hypertension-related models. As briefly mentioned previously, in male spontaneously hypertensive rats, we have demonstrated previously that compound 21 acutely evokes vasodepressor responses during simultaneous AT_1R blockade.[14] However, the long-term and sex-specific responses to direct AT_2R stimulation during hypertension in the presence and absence of combined AT_1R blockade have not been investigated previously. Recently, Matavelli et al[26] reported that compound 21, over 4 days, reduced renal inflammation and increases renal NO-cGMP levels in 2-kidney, 1-clip Goldblatt hypertensive rats, in the absence of any reductions in arterial pressure.

In conclusion, we have shown in both male and female normotensive rats that compound 21 produces renal vasodilatory and natriuretic effects via direct stimulation of the AT_2R, in the absence of AT_1R blockade. Given its significant influence on renal function, the AT_2R may therefore represent a significant therapeutic target for the treatment of renal and cardiovascular disease. Moreover, compound 21 should be considered as an ideal therapeutic tool to further elucidate the sex-specific potential of direct AT_2R stimulation as a target in the treatment of renal and cardiovascular disease.

Perspectives

Taken together, these findings strongly suggest a protective role of the AT_2R in the kidney and vasculature. The AT_2R appears to play a pivotal role in the regulation of renal hemodynamics and excretory function, which is enhanced in females. Future studies may therefore investigate the beneficial effects of AT_2R stimulation in hypertensive states and may ultimately lead to the development of sex-specific therapies.

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Disclosures

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