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V_1/V_2 Vasopressin receptor antagonism potentiates the renoprotection of renin–angiotensin system inhibition in rats with renal mass reduction

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Blockade of the renin–angiotensin system (RAS), the standard treatment for chronic proteinuric nephropathy, slows but may not halt progression of the disease, particularly when therapy is started late. Because vasopressin may also play a role in the progression of renal disease, we measured the effect of a dual V_{1a} and V_2 vasopressin receptor antagonist (RWJ-676070) alone or combined with angiotensin-converting enzyme inhibition or angiotensin II type 1 receptor blockade on proteinuria and renal disease progression during overt nephropathy. Twenty-one days after renal mass reduction, a time of established injury, rats were given vehicle, RWJ-676070, enalapril, losartan, RWJ-676070 plus enalapril, or losartan in drinking water for an additional 39 days. RWJ-676070 returned the blood pressure to pre-treatment levels, which were significantly lower than those in vehicle-treated rats. Enalapril, losartan, and the combined therapies reduced blood pressure to a greater extent. RWJ-676070 afforded a partial antiproteinuric effect, which was enhanced by the addition of enalapril or losartan. Renal functional impairment, and glomerular and tubular changes were partially ameliorated by RWJ-676070; parameters significantly improved with either enalapril or losartan alone and improved to a greater extent with the combined therapies. Our findings suggest that vasopressin receptor antagonists could be of additional therapeutic value in the treatment of chronic proteinuric nephropathy.

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Chronic kidney diseases are increasing worldwide and are emerging as a global threat to human health.[1] Progression to end-stage renal disease is the final common pathway of many forms of glomerular disease independently of the initial insult.[2] In the past two decades, research in animals and humans has helped our understanding of the mechanisms of how chronic kidney diseases progress and has indicated possible preventive maneuvers.[2,3] These studies have established that the progressive deterioration of renal function is the result of compensatory glomerular hemodynamic changes in response to nephron loss due to the original insult that, in turn, causes relentless injury of remaining intact nephrons.[3] Glomerular capillary hypertension, originally serving to maintain ultrafiltration despite fewer nephrons, impairs the barrier's size-selective function and causes excessive protein ultrafiltration in animal models. It has been suggested that, rather than simply a marker of damage, abnormally ultrafiltered proteins can be toxic to the kidney by exerting a nephritogenic effect that would favour tissue scarring and functional impairment.[4,5]

Current treatment for proteinuric chronic nephropathies is based on blockade of the renin–angiotensin system (RAS) with angiotensin-converting enzyme (ACE) inhibitors and/or angiotensin type 1 receptor blockers (ARBs) that limit proteinuria and reduce glomerular filtration rate decline and risk of end-stage renal disease more effectively than other antihypertensive treatments.[6] Full remission of the disease, however, is seldom achieved particularly when pharmacological intervention is started late. Thus, a significant reduction of the incidence of end-stage renal disease is likely to be obtained, provided we can improve the current degree of renoprotection. This goal may be attainable with a more complex strategy than with a single pharmacological intervention on the RAS.

In addition to the RAS, vasopressin has also been suggested to have a role in the progression of chronic kidney disease by increasing intraglomerular capillary pressure[7] and stimulation of mesangial cell proliferation.[8] Vasopressin exerts a variety of biological effects by specific G-protein-coupled

receptor subtypes, including vascular V_1, renal V_2, and hormone-releasing pituitary V_3 receptors.[9] Recently, orally active nonpeptide vasopressin receptor antagonists have been developed, the potential therapeutic uses of which include arterial hypertension, hyponatremia, and congestive heart failure.[10–12] Thus, V_1 and/or V_2 receptor antagonists could theoretically be included in a multimodal strategy to implement renoprotection.

This study was designed with the aims to assess (1) the effect of a dual V_{1a} and V_2 vasopressin receptor antagonist RWJ-676070[13,14] on proteinuria and renal disease progression in rats with renal mass reduction (RMR) starting when animals had overt nephropathy; and (2) the renoprotective effect of adding-on V_1/V_2 receptor antagonist to either the ACE inhibitor enalapril or the ARB losartan.

Renal mass reduction in rats, which mimics the human condition of nephron loss, is characterized by severe hypertension, proteinuria, glomerulosclerosis, and tubulointerstitial damage, associated with progressive renal functional deterioration.[15] Although ACE inhibition is fully effective in this model when treatment is started soon after surgical ablation,[16] only partial renoprotection is afforded when animals receive the ACE inhibitor at a phase of established overt disease.[17,18] Similarly, in the same model only partial benefit can be achieved with ARB at the stage of overt nephropathy.[18]

RESULTS

Mortality

By the end of the study, 6 out of the 20 rats with RMR died in the vehicle group, 5 out of 20 in the group given V_1/V_2 antagonist alone, 2 out of 20 in the group given enalapril alone, 2 out of 12 in the group given losartan alone, 3 out of 20 and 1 out of 12 in the group receiving the combination of V_1/V_2 antagonist plus enalapril or losartan, respectively. In the control group, all rats were alive.

Body weight and food intake

In all experimental groups, the animals gained weight with time (Table 1). However, in the RMR rats the mean values of body weight were lower than those of controls during the study period. Thus, at day 21 after surgery, before treatment, body weight of RMR rats ($n = 104$) and controls ($n = 14$) averaged 367 ± 5 and 429 ± 11 g, respectively.

At the end of the study, the mean body weight of rats given vehicle or RWJ-676070 was significantly ($P < 0.01$) lower than that of control animals. In the group of rats treated with the combination of RWJ-676070 plus enalapril or losartan, values were numerically higher than those in RMR animals treated with single drugs (Table 1).

As shown in Table 1, the mean values of total food intake of RMR rats given vehicle or V_1/V_2 antagonist were numerically lower than those of control rats. Food intake of RMR rats given enalapril or losartan alone, or combined with V_1/V_2 antagonist was similar to that of controls.

Diuresis and water intake

As compared with controls, diuresis was more than double in RMR rats 21 days after surgery, before treatment (45 ± 1 versus 22 ± 2 ml/24 h, $P < 0.01$), and remained significantly ($P < 0.01$) higher until day 60 (Table 1). No statistical difference was detected among the groups of RMR rats during the study. Of note is the fact that in rats given the V_1/V_2 antagonist alone or in combination with enalapril or losartan, the daily urine volume was not further increased as compared with animals receiving vehicle. In parallel with the increase in diuresis, the water intake was significantly ($P < 0.05$) enhanced in all RMR groups as compared with controls (Table 1).

Serum and urinary electrolytes

Serum Na levels measured at day 60 were similar in RMR groups and controls, with mean values ranging from 147 ± 0.8 to 151 ± 2 mEq/l. At the same time point also serum K concentration was comparable in all study groups (average: 6 ± 0.1 to 6 ± 0.3 mEq/l). As shown in Table 2, the urinary Na excretion rate was numerically lower in RMR rats given vehicle as compared with controls. RWJ-676070 numerically, but not significantly, increased urinary Na excretion. Actually, the mean values were comparable with those in control rats. Similarly, enalapril or losartan treatment normalized the urinary Na excretion. The combined therapies did not result in any further change in Na excretion as compared with the single treatments. Urinary Na concentration in RMR rats was significantly ($P < 0.01$) lower than control group (Table 2). The mild natriuretic effect of the V_1/V_2 antagonist is also supported by the minimal increase in urinary Na concentration in RMR rats

Table 1 | Body weight, food intake, diuresis, and water intake evaluated in rats with renal mass reduction (RMR) at 60 days

Group	Body weight (g)	Food intake (g/24 h)	Diuresis (ml/24 h)	Water intake (ml/24 h)
RMR				
Vehicle	437 ± 17	22 ± 2	47 ± 3	64 ± 6
RWJ-676070	457 ± 18	24 ± 2	45 ± 4	68 ± 8
Enalapril	483 ± 12	27 ± 1	43 ± 2	63 ± 3
Losartan	478 ± 14	29 ± 1*	45 ± 4	75 ± 5
RWJ-676070+enalapril	490 ± 9	29 ± 1*	42 ± 3	68 ± 4
RWJ-676070+losartan	512 ± 16*	28 ± 2	41 ± 2	73 ± 6
Control	542 ± 16	27 ± 1	22 ± 1	41 ± 2

Values are expressed as mean ± s.e. *$P < 0.05$ vs RMR+vehicle. Statistical analysis comparing the control group and the RMR groups is reported in the text.

Table 2 | Effect of V_1/V_2 antagonist on urinary Na excretion and concentration in renal mass reduction (RMR) rats

Group	Urinary Na excretion (mEq/day)	Urinary Na concentration (mEq/l)
RMR		
Vehicle	2.6 ± 0.2	57 ± 4
RWJ-676070	3.1 ± 0.2	73 ± 6
Enalapril	3.2 ± 0.1	76 ± 5
Losartan	3.3 ± 0.2	78 ± 8
RWJ-676070+enalapril	3.3 ± 0.2	75 ± 3
RWJ-676070+losartan	3.0 ± 0.1	76 ± 6
Control	3.3 ± 0.2	152 ± 10

Values are expressed as mean ± s.e. Statistical analysis comparing the control group and the RMR groups is reported in the text.

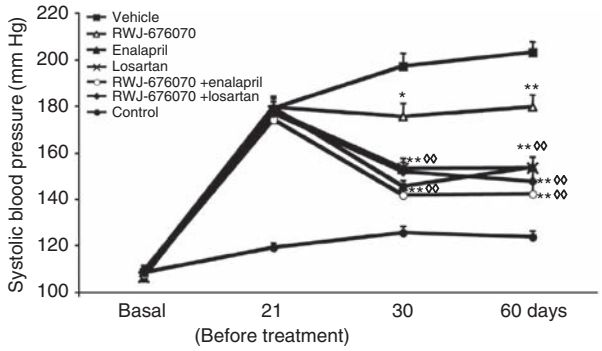

Figure 1 | Time course of systolic blood pressure (SBP) in rats with renal mass reduction (RMR) administered vehicle, the V_1/V_2 antagonist RWJ-676070, enalapril, losartan, RWJ-676070 plus enalapril or losartan. Treatment was started 21 days after surgical ablation, when RMR rats developed hypertension ($P<0.01$ vs control). Data are mean ± s.e. *$P<0.05$, **$P<0.01$ vs vehicle; ◇$P<0.05$, ◇◇$P<0.01$ vs RWJ-676070. Statistical analysis comparing the control group and the RMR groups is reported in the text.

given the compound compared with those receiving the vehicle alone (Table 2), despite similar daily urine volume.

Systolic blood pressure

The time course of systolic blood pressure (SBP) is shown in Figure 1. Rats with RMR showed a significant ($P<0.01$) increase in SBP as compared with controls at day 21 post-surgery (before starting treatment). In rats given vehicle SBP further increased during the study ($P<0.01$ versus control). Treatment with RWJ-676070 kept SBP at values similar to pretreatment and significantly lower than those measured in RMR given vehicle during the 60-day follow-up. Enalapril, losartan, and the combined therapies reduced SBP to a greater extent than V_1/V_2 antagonist alone. In the group of RMR rats given RWJ-676070 plus enalapril, SBP values were not different from those of control group during the all study.

Urinary protein excretion

As shown in Figure 2, at day 21 after surgery, all RMR rats developed proteinuria ($P<0.01$ versus control) that further

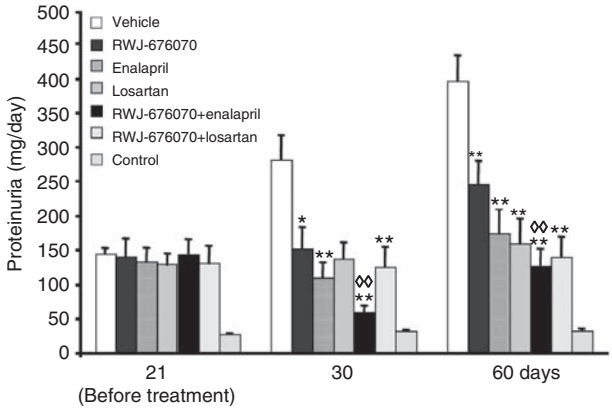

Figure 2 | Time course of urinary protein excretion in rats with RMR treated from day 21 after surgery with vehicle, RWJ-676070, enalapril, losartan, RWJ-676070 plus enalapril or losartan. Data are mean ± s.e. *$P<0.05$, **$P<0.01$ vs vehicle; ◇◇$P<0.01$ vs RWJ-676070 alone. Statistical analysis comparing the control group and the renal mass reduction (RMR) groups is reported in the text.

increased during time in the vehicle group. Treatment with RWJ-676070 caused a 46% reduction of proteinuria with respect to vehicle at day 30 ($P<0.05$); proteinuria values of V_1/V_2 antagonist-treated rats remained numerically lower than those of vehicle at day 60 (38% reduction). Enalapril therapy alone maintained significantly ($P<0.01$) lower levels of proteinuria over time than those of vehicle. Combined administration of V_1/V_2 antagonist with enalapril resulted in a further reduction of urinary protein excretion as compared with mean values in RMR rats given V_1/V_2 antagonist or enalapril alone. Notably, at day 30, proteinuria values were fairly comparable with those measured in control group. Losartan alone kept proteinuria at levels similar to pretreatment and numerically lower than those of vehicle-treated rats at day 30; values became significantly different at day 60 ($P<0.01$). In rats given V_1/V_2 antagonist plus losartan, the mean proteinuria levels were numerically lower than those measured in rats given each agent alone.

Renal function

Renal function progressively declined in RMR rats given vehicle, as indicated by serum creatinine levels that significantly increased over controls (day 60: 1.91 ± 0. 20 vs 0.50 ± 0 mg per 100 ml, $P<0.01$). Treatment with V_1/V_2 antagonist partially prevented the increase in serum creatinine levels, although a statistical significance was not achieved (Figure 3). In RMR rats administered enalapril or losartan alone, serum creatinine levels were significantly ($P<0.01$) lower than those in rats given vehicle, but still higher ($P<0.01$) than in control group. A further reduction in serum creatinine concentration was achieved in rats given the combined therapies ($P<0.05$ versus control). Overall, a significant correlation was found between renal function, serum creatinine, and urinary protein excretion rate ($r=0.798$, $P<0.001$) (Figure 4).

Renal histology and inflammatory cell infiltrate in the interstitium

Table 3 reports the results of renal morphological analysis by light microscopy performed at day 60. RMR rats given vehicle showed glomerulosclerosis affecting on average 62% of glomeruli ($P<0.01$ versus control). Lesions were characterized by segmental areas of sclerosis and hyalinosis with capillary collapsing and adhesion to Bowman's capsule. Tubular damage was also documented, consisting of luminal proteinaceous casts and tubular atrophy (score: 1.4 ± 0.1, $P<0.01$ versus control). In RMR rats treated with RWJ-676070, the glomerular lesions were less diffuse and severe and the percentage of glomeruli with sclerotic changes averaged 41% ($P<0.05$ versus vehicle; $P<0.01$ versus control). Tubular damage was also mildly reduced. Enalapril treatment was associated with a partial but significant reduction in the percentage of glomeruli with sclerosis (on average 25%; $P<0.01$ versus vehicle; $P<0.01$ versus control). The combined administration of V_1/V_2 antagonist and enalapril further lowered the percentage of sclerotic glomeruli to 16% ($P<0.01$ versus vehicle or V_1/V_2 antagonist, not significant versus control) and also significantly ameliorated tubular damage ($P<0.05$ versus vehicle). In rats given losartan, the percentage of glomeruli with sclerotic changes averaged 32% ($P<0.01$ versus vehicle; $P<0.01$ versus control). Glomerulosclerosis decreased to 23% after the addition of V_1/V_2 antagonist to losartan ($P<0.01$ versus vehicle; $P<0.05$ versus control). Tubular damage was mildly affected by either losartan alone or the combined therapy ($P<0.01$ versus control).

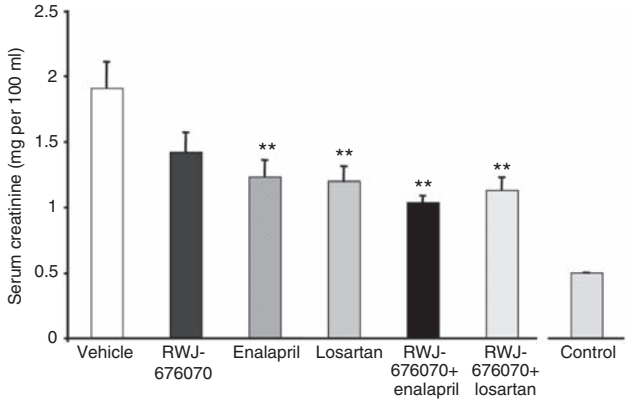

Figure 3 | Serum creatinine at day 60 in rats with renal mass reduction (RMR) administered vehicle, RWJ-676070, enalapril, losartan, RWJ-676070 plus enalapril or losartan. Data are mean ± s.e. $^{**}P<0.01$ vs vehicle. Statistical analysis comparing the control group and the RMR groups is reported in the text.

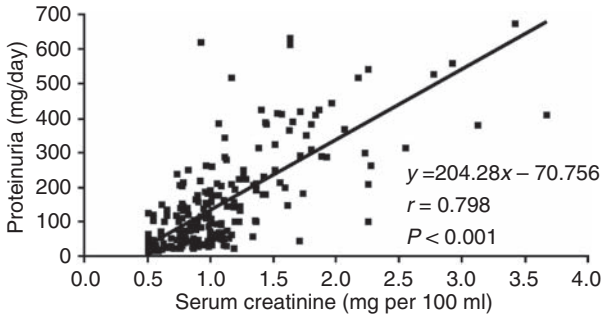

Figure 4 | Correlation between renal function (measured as serum creatinine levels) and proteinuria evaluated in all the experimental groups considering values at baseline and at days 30 and 60 during treatment.

As shown in Table 3, a large accumulation of ED-1-positive monocytes/macrophages was found in the renal interstitium of RMR rats given vehicle ($P<0.01$ versus control). In all groups of RMR-treated rats, the number of ED-1-positive cells were higher ($P<0.01$) than that of control group. RWJ-676070 reduced interstitial infiltrates by 24% with respect to vehicle-treated rats. Enalapril and losartan were more effective in limiting to a significant extent the number of ED-1-positive cells ($P<0.01$ versus vehicle). The degree of cell infiltrate in the interstitium was decreased further by the combination of V_1/V_2 antagonist and enalapril, but not with losartan.

Plasma levels of V_1/V_2 antagonist in RMR rats

At the end of the study, blood samples were collected to measure plasma concentration of RWJ-676070 in animals given the drug alone or in combination with the ACE inhibitor. Plasma levels of the V_1/V_2 antagonist in RMR rats given the drug alone averaged 2094 ± 394 ng/ml. In RMR rats on V_1/V_2 antagonist plus enalapril, the mean plasma level of the compound was numerically, but not significantly, lower

Table 3 | Renal histology and interstitial accumulation of monocytes/macrophages

Group	Glomeruli with sclerotic changes (%)	Tubular damage (score)	ED-1-positive cells (cells/HPF)
RMR			
Vehicle	62 ± 5	1.4 ± 0.1	62 ± 3
RWJ-676070	41 ± 6*	1.1 ± 0.1	47 ± 5
Enalapril	25 ± 5**	0.9 ± 0.1	39 ± 4**
Losartan	32 ± 6**	1 ± 0	37 ± 6**
RWJ-676070+enalapril	16 ± 3**◇◇	0.8 ± 0.1*	32 ± 3**
RWJ-676070+losartan	23 ± 5**	1.1 ± 0.1	38 ± 6**
Control	0	0	12 ± 1

HPF, high-power field; RMR, renal mass reduction.
Values are expressed as mean ± s.e. $^{*}P<0.05$, $^{**}P<0.01$ vs RMR+vehicle; $^{◇◇}P<0.01$ vs RMR+RWJ-676070.
Statistical analysis comparing the control group and the RMR groups is reported in the text.

than that in animals given the V_1/V_2 antagonist alone (1464 ± 261 ng/ml).

DISCUSSION

We found that the V_1/V_2 antagonist RWJ-676070[13] reduced systemic blood pressure, afforded partial antiproteinuric effect, and ameliorated glomerular and tubular damage, ultimately slowing renal function deterioration, if administered as a single agent to rats with RMR. Of note, the therapy was started when animals already had overt nephropathy with the aim to interfere with the disease process.

The renoprotective effect of chronic vasopressin receptor blockade with nonpeptide orally active compounds has been previously documented in several experimental rat models of progressive kidney diseases, including adriamycin nephropathy,[19,20] spontaneously hypercholesterolemic rats undergoing unilateral nephrectomy,[21] streptozotocin-induced diabetes mellitus,[22] and partially nephrectomized, salt-loaded spontaneously hypertensive rats.[23] In all instances, however, treatment with vasopressin receptor antagonists was started at the time of induction of kidney disease to prevent the development of proteinuria and renal structural injury, and possibly progressive renal function deterioration. In this study, we showed that the selective blockade of V_1/V_2 receptors had therapeutic efficacy if started 3 weeks after five-sixths nephrectomy when overt nephropathy was already manifested. In a previous report in rats with five-sixths renal mass ablation, a V_{1a}-antagonist ameliorated renal disease progression when given 2 weeks after surgery, but was uneffective when treatment was given as a later intervention at 6 weeks.[24] In this study, the drug was administered daily by gavage, not in the drinking water as we did, therefore its effect could be more short-lived. More complete antagonism of vasopressin activity with the combined V_1/V_2 compound could explain the renoprotection we documented. The present findings open the possibility that nonpeptide vasopressin receptor antagonists could be of additional therapeutic value as renoprotective agents in patients with proteinuric chronic nephropathies. Whether the beneficial effect of the V_1/V_2 receptor antagonist was sustained by blockade of V_1, V_2 or both receptors, and how receptor antagonism afforded renoprotection remains ill defined. In a rat model of progressive nephropathy induced by adriamycin and accelerated by deoxycorticosterone acetate-salt hypertension, the V_1 antagonist (OPC-21268) as well as the V_2 antagonist (OPC-31260) significantly reduced proteinuria, as well as glomerular and tubulointerstitial injury as compared with the untreated animals.[20] Moreover, in streptozotocin-induced diabetic rats, the development of albuminuria was completely prevented by chronic antagonism of V_2-mediated actions of vasopressin.[22] The importance of V_1/V_2 receptor antagonism in renoprotection is also underlined by the observation that in Brattleboro rats, deficient in vasopressin, submitted to five-sixths nephrectomy, the administration of the V_2 receptor agonist DDAVP accelerated the progression of chronic kidney disease more than that of vasopressin, an agonist of both V_1 and V_2 receptors.[25]

There is evidence showing that the activation of V_1 receptors may contribute to glomerular damage by inducing contraction of mesangial cells,[26,27] and vasoconstriction of glomerular efferent arterioles which enhances glomerular capillary pressure.[7] Studies have suggested that the activation of V_2 receptors at tubular level inhibits tubuloglomerular feedback as a result of reduction in salt concentration at the macula densa, secondary to V_2-receptor-mediated urine concentrating process.[28,29] Blockade of tubuloglomerular feedback translates into the elevation of intraglomerular capillary pressure and eventually glomerular injury.[30] In addition, the evidence of a direct V_2 effect on glomerular hemodynamics is also available, as shown by the fact that the infusion of the V_2 antagonist OPC-31260 to hydropenic rats given the vasopressin-V_2 receptor agonist desmopressin, normalized glomerular filtration rate compared with vehicle.[31] Therefore, the glomerular mechanisms of actions of V_1 and V_2 receptors would imply that renoprotection afforded by RWJ-676070 in our model can result from its ability to block both V_1 and V_2 receptors causing reduction of intraglomerular capillary pressure and eventually lowering abnormal protein traffic through the glomerular capillary barrier, thus limiting urinary protein excretion and renal scarring.[32]

Interestingly, in RMR rats receiving the V_1/V_2 antagonist, the daily urine volume was not further increased as compared with RMR animals receiving the vehicle. The lack of a diuretic effect of the V_1/V_2 antagonist could be explained by the fact that in this rat model the tubular water reabsorption by the collecting duct principal cells is already largely reduced due to a marked reduction in aquaporin 2 and 3 expression, which translates in a marked increase in the daily urine volume,[33] as we found in RMR rats given vehicle. In this setting, the effect on water reabsorption of the V_1/V_2 antagonist might be negligible, if any. This would result in no significant change in the diuresis as compared with animals receiving the vehicle. Alternatively, but not exclusively, the possibility exists that the V_2 antagonistic actions at tubular level of the tested V_1/V_2 antagonist may be very weak.

On the other hand, we found that V_1/V_2 antagonism numerically increased the urinary Na excretion as compared with vehicle in RMR rats. This finding indicates that the V_1/V_2 antagonist inhibited the mild Na tubular reabsorption occurring in rats undergoing renal mass ablation. This effect could be attributed to the antagonism of V_2 receptor at the collecting duct. Indeed, evidence is available that in the rats the V_2 agonist dDAVP increased mRNA expression of the β- and γ-subunits of the endothelial sodium channel in renal collecting ducts.[34] This was associated with marked increase in sodium reabsorption in response to exogenous dDAVP *ex vivo*.[34] The mild natriuretic effect of the V_1/V_2 antagonist is also supported by the minimal increase in urinary Na concentration in RMR rats given the V_1/V_2 antagonist

compared with those given vehicle alone, despite similar urine volume.

A major finding in this study is that the combined administrations of the V_1/V_2 antagonist with RAS inhibitors were more effective than either treatment alone in protecting animals from renal damage. Actually, 68% reduction in urinary protein excretion rate was found in RMR rats after combined treatment with V_1/V_2 antagonist and the ACE inhibitor enalapril with respect to RMR vehicle-treated animals, as compared with a 38 and 56% reduction observed with V_1/V_2 antagonist and enalapril alone, respectively. Renal function impairment and structural changes of RMR rats were also consistently ameliorated by the combined therapy. The effect of the combined treatment with V_1/V_2 antagonist and ACE inhibitor on proteinuria, renal function, and structure was also higher than that with ACE inhibitor alone, although the difference did not reach statistical significance. Renoprotection was less effective combining V_1/V_2 antagonist with the ARB losartan than with enalapril, despite comparable blood pressure control.

Pharmacological inhibition of RAS with ACE inhibitors and ARBs has been shown in the landmark experimental and clinical studies to limit proteinuria and attenuate decline in renal function inexorably associated with chronic renal diseases.[35,36] These effects have been attributed to the control of systemic and intraglomerular hypertension along with the ability of this class of drugs to limit excess protein ultrafiltration and its deleterious consequences.[2] When given soon after disease induction, ACE inhibitors consistently limit hypertension, proteinuria, and renal injury in virtually all animal models of renal disease.[16,37–39] By contrast, when treatment starts late in the course of the disease at the stage of overt nephropathy, drugs that antagonize RAS are not uniformly effective.[40–42] Most patients with proteinuric nondiabetic or diabetic renal disease are actually referred late or very late to the nephrologist, and in such circumstances treatment with ACE inhibitors may be of relatively little value to control disease progression. To model the human condition, we started the combined treatment with RAS blockers and the V_1/V_2 receptor antagonist in rats with renal mass ablation at the stage of overt nephropathy. This setting was instrumental to demonstrate for the first time that the combined therapy did retain therapeutic effectiveness, when RAS inhibitor alone was no longer enough. Suppression of the RAS after treatment with either ACE inhibitors or ARBs remains incomplete.[43] A key reason for this is that these therapies stimulate a reactive increase in renin activity,[44] because they disrupt the short feedback loop by which angiotensin II normally inhibits the release of renin from the kidney.[45] The reactive plasma renin stimulation by ACE inhibitors, particularly after long-term use, provides an explanation for why current RAS inhibitors are sometimes suboptimal or not effective. Recent evidence indicates that in mice knockout for V_{1a} receptor the expression of renin in granule cells of the macula densa was reduced, which led to a decreased level of plasma renin.[46] These findings would imply that the additional renoprotective effect we observed by combining ACE inhibitor with the V_1/V_2 receptor antagonist in rats with renal mass ablation could be due to a renin defect secondary to blockade of V_{1a} receptor by RWJ-676070. However, prolonged treatment with the combined drugs showed that after the initial decline in proteinuria as compared with baseline value, there was an escape of the antiproteinuric effect on the long term, similar to that documented with ACE inhibitor alone. This suggests that the renoprotection afforded by RWJ-676070 through inhibition of renin synthesis is negligible, if any. Thus, the further beneficial effect of adding-on the V_1/V_2 receptor antagonist to RAS inhibitors on renal disease progression could likely be related to an independent direct effect of the compound through blockade of V_1 and V_2 glomerular receptors. As additional complementary mechanism, the increase in salt concentration at macula densa secondary to tubular V_2 receptor antagonism would result into activation of tubuloglomerular feedback and further reduction of intraglomerular capillary pressure.

In conclusion, this study showed that in a severe model of progressive nephropathy only partially responsive to RAS blockade, combining the V_1/V_2 receptor antagonist RWJ-676070 with RAS inhibitors may further potentiate the renoprotective effect of RAS blockade alone. Possible targets of the combined agents' action are the abnormal glomerular hemodynamics and the related perm-selective dysfunction, and indirectly the secondary pathways of glomerular and tubular interstitial damage triggered by glomerular and tubular protein overload, which act to perpetuate renal injury in rats with RMR and overt nephropathy. The proposed therapeutic approach could add to the available armamentarium for animals with advanced renal disease in which RAS inhibitors alone fail to fully prevent progressive renal injury.

MATERIALS AND METHODS

Animals and experimental design

Male Sprague–Dawley, CD-COBS rats (Charles River, Calco, Italy), with initial body weights of 275–300 g were used. Animal care and treatment were conducted in accordance with the institutional guidelines that are in compliance with national (Decreto Legislativo n.116, Gazzetta Ufficiale suppl 40, 18 febbraio 1992, Circolare n.8, Gazzetta Ufficiale 14 luglio 1994) and international laws and policies (EEC Council Directive 86/609, OJL358-1, December 1987; Guide for the Care and Use of Laboratory Animals, U.S. National Research Council, 1996). All animals were housed in a room in which the temperature was kept constant on a 12-h dark/12-h light cycle and allowed free access to standard diet containing 20% protein by weight and tap water. RMR was obtained by right nephrectomy and ligation of 2 or 3 branches of the main renal artery, according to Olson.[15] Twenty-one days after surgery, when rats had hypertension and proteinuria, they were allocated to receive the following treatments in the drinking water: group 1 ($n=20$) rats given vehicle (water); group 2 ($n=20$) rats given the V_1/V_2 receptor antagonist RWJ-676070 (Johnson & Johnson Pharmaceutical, Raritan, NJ, USA),[13,14] at the daily dose of 30 mg/kg; group 3

($n=20$) rats given the ACE inhibitor enalapril (Sigma-Aldrich, St Louis, MO, USA) (15 mg/l);[38] group 4 ($n=12$) rats given the ARB losartan (Merck Sharp & Dohme, Rome, Italy) (10 mg/kg); group 5 ($n=20$) rats treated with the combination of the V_1/V_2 antagonist and enalapril; and group 6 ($n=12$) rats treated with the combination of the V_1/V_2 antagonist and losartan. An additional group of sham-operated rats (group 7, $n=14$) without any treatment was followed for the same period as controls.

In all groups, SBP was measured at baseline, day 21 after surgery (before treatment) and day 30 and 60 during treatment, by the tail-cuff method.[47] Urinary protein excretion was monitored at the same times. Serum creatinine concentration, as an index of renal function, was measured at baseline, day 30 and 60. At day 60, serum concentration of Na and K and urinary excretion of Na were determined. Plasma levels of the V_1/V_2 antagonist were also measured (at Johnson & Johnson). Rats were then killed, the kidneys were removed and renal tissue was processed for morphological and immunohistochemical studies.

Biochemical parameters

Twenty-four hour urine samples were collected using metabolic cages, and proteinuria was determined by modified Coomassie blue G dye-binding assay for proteins with bovine serum albumin as standard.[48.] Serum creatinine levels were measured by the Refloron test (Roche Diagnostics, Indianapolis, IN, USA). Serum and urinary electrolytes were measured using an autoanalyzer (CX5, Beckman Instruments, Fullerton, CA, USA).

Renal histology and immunohistochemistry

The removed kidneys were fixed overnight in Duboscq-Brazil, dehydrated in alcohol, and embedded in paraffin. Kidney samples were sectioned at 3 μm intervals and the sections were stained with Masson's trichrome, hematoxylin and eosin, and periodic-acid Schiff reagent. Tubular changes (atrophy, casts, and dilatation) were graded from 0 to 4+ (0, no change; 1+, changes affecting less than 25% of the sample; 2+, changes affecting 25–50% of the sample; 3+, changes affecting 50–75% of the sample; 4+, changes affecting 75–100% of the sample). Data are expressed as the mean score values for tubular damage for each animal. At least 100 glomeruli were examined for each animal and the extent of glomerular damage was expressed as the percentage of glomeruli presenting sclerotic lesions. All renal biopsies were analyzed by the same pathologist who was unaware of the nature of the experimental groups.

Detection of ED-1 antigen was performed on paraffin sections using a mouse monoclonal antibody (Chemicon, Temecula, CA, USA) by an alkaline phosphatase-Fast Red technique as previously described.[49] Positive cells were counted in at least 10 randomly selected high-power microscopic fields (×400) per each animal.

Statistical analysis

Data are expressed as mean ± s.e. Data were analyzed by analysis of variance with Bonferroni correction or by the nonparametric Kruskal–Wallis test for multiple comparisons. Correlation between serum creatinine levels and proteinuria was calculated by a linear regression analysis. The statistical significance level was defined as $P<0.05$.

DISCLOSURE

LH is an employee of Johnson & Johnson Pharmaceutical Research Institute. All the other authors declared no competing interests.

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