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Long-term regulation of proximal tubule acid–base transporter abundance by angiotensin II

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In the proximal tubule, angiotensin II (Ang-II) regulates HCO_3^- reabsorption and H^+ secretion by binding the type 1 Ang-II (AT1) receptor, stimulating Na^+/HCO_3^- cotransport and Na^+/H^+ exchange. Studies were carried out to determine if long-term changes in Ang-II receptor occupation alter the abundance of the basolateral Na^+/HCO_3^- cotransporter (NBC1) or the apical membrane type 3 Na^+/H^+ exchanger (NHE3). In the first set of experiments, rats eating a low-sodium diet were infused with the AT1 blocker, candesartan, or vehicle. In the second, lisinopril-infused rats were infused with either Ang II or vehicle. Transporter abundances were determined in whole kidney homogenates (WKH) and in brush border membrane (BBM) preparations by semiquantitative immunoblotting. Tissue distribution of transporters was assessed by immunocytochemistry. Blockade of the AT1 receptor by candesartan caused decreased abundance of NBC1 in WKH ($59 \pm 9\%$ of control; $P < 0.05$) and Ang-II infusion increased abundance ($130 \pm 7\%$ of control; $P < 0.05$). Changes in NBC1 in response to candesartan were confirmed immunohistochemically. Neither candesartan nor Ang II infusion affected the abundance of NHE3 in WKH or cortical homogenates. Candesartan decreased type 2 sodium-phosphate cotransporter abundance in both WKH ($52 \pm 7\%$ of control; $P < 0.05$) and BBM ($32 \pm 7\%$ of control; $P < 0.05$). Serum bicarbonate was decreased by candesartan and increased by Ang-II. Candesartan also decreased urinary ammonium excretion ($P < 0.05$). The long-term effects of Ang-II in the proximal tubule may be mediated in part by regulation of NBC1 abundance, modifying bicarbonate reabsorption.

Angiotensin II (Ang-II) regulates acid–base transport in the proximal tubule by stimulating active apical H^+ secretion, apical $Na \pm H^+$ exchange,[1–3] and basolateral Na^+/HCO_3^- cotransport[4] as well as by enhancing ammonium secretion[5] in response to acid loading. In addition, Ang-II helps to maintain sodium balance during periods of dietary sodium restriction or contraction of extracellular fluid volume[6] by increasing fluid and NaCl reabsorption in the proximal tubule.[7,8] Ang-II produces its effects, at least in part, due to direct action in the renal tubule.[9] Those effects are mediated by binding to the type 1 Ang-II (AT1) receptor[10,11] and are seen at lower Ang-II concentrations than are needed for short-term systemic hemodynamic actions.[9] AT1 receptors are also expressed in the collecting duct,[12] where they regulate epithelial Na channel activity[13] and abundance.[14]

Regulation of renal tubule H^+, HCO_3^-, and Na^+ transport by Ang-II has been investigated largely in relatively short-term experiments with observations made within a few minutes of Ang-II addition.[1,4,7,8,15] However, there is growing evidence that a variety of mediators of transport regulation in the kidney, such as vasopressin[16] and aldosterone,[17] work by both short-term and long-term actions. Long-term actions are associated with increases in abundances of transporter proteins, whereas short-term actions are associated with regulated trafficking as well as post-translational modifications of the transporter proteins.

Approximately 70% of filtered sodium is reabsorbed in the proximal tubule. A large portion of the apical component of sodium, proton, and ammonium transport is mediated by the type 3 $Na \pm H^+$ exchanger (NHE3).[18] A much smaller fraction of the total sodium absorbed enters the cell along with phosphate ion on the type 2 sodium-phosphate cotransporter (NaPi-2), the abundance and activity of which changes in response to chronic acid–base disturbances.[19] In the basolateral membrane, the type 1 sodium-bicarbonate cotransporter (NBC1) carries sodium against an electrochemical gradient owing to its coupling with bicarbonate anion.[20]

In this study, we used antibodies to NBC1, NHE3, and NaPi-2 to investigate whether long-term changes in Ang-II receptor occupation are associated with changes in abundance of these transporters. This is the first study that addresses the long-term effects of changes in AT1 receptor

occupation on the abundance of NBC1 protein and mRNA levels. Two different experimental protocols were used to manipulate the level of AT1 receptor occupation by Ang-II.

RESULTS
Physiological parameters

Table 1 details urine and plasma measurements in both the candesartan and the Ang-II infusion experiments. Urinary sodium excretion rates indicate that there were no sustained differences between control and experimental data, indicating that the animals had reached a steady state such that excretion largely matched the fixed intake of solutes and water. Potassium excretion, however, was slightly lower in candesartan rats versus controls. In both studies, serum aldosterone levels were low relative to the K_d of the mineralocorticoid receptor $(1.2 \, \text{nM})$[21] and were not significantly affected by the experimental treatments (Table 1).

Both experimental treatments significantly affected systemic acid–base status. Candesartan treatment caused a decrease in plasma bicarbonate concentration from control values of 26 ± 1 to $20 \pm 1 \, \text{mM}$ $(P < 0.05)$. Ang-II infusion had the opposite effect on plasma bicarbonate, causing an increase from $25 \pm 0 \, \text{mM}$ in control rats to $28 \pm 1 \, \text{mM}$ in Ang-II-treated animals $(P < 0.05)$. Urinary ammonium excretion was also lower $(P < 0.03)$ in candesartan-treated rats $(0.73 \pm 0.07 \, \text{mmol/day})$ than in controls $(0.97 \pm 0.06 \, \text{mmol/day})$ (Table 1).

Effects on NBC1 expression

Transport of bicarbonate across the basolateral plasma membrane in the proximal tubule is mediated by NBC1.[22] To test whether AT1 receptor blockade with candesartan alters NBC1 expression, we carried out semiquantitative immunoblotting (Figure 1). In the setting of a moderately reduced NaCl diet (0.5 meq/200 g body weight/day NaCl), candesartan administration resulted in a marked decrease in

renal NBC1 abundance, reducing NBC1 band density to $59 \pm 9\%$ of control. To address further the role of Ang-II in regulation of NBC1 abundance, we infused Ang-II into rats in which the basal levels of Ang-II was suppressed by administration of the angiotensin-converting enzyme inhibitor, lisinopril. As can be seen in Figure 1, Ang-II infusion was associated with a significant increase in NBC1 band density to $130 \pm 7\%$ of control. Immunoperoxidase immunocytochemistry (Figure 2) confirmed that this antibody selectively labels proximal tubules. The general distribution of labeling was unchanged in response to candesartan administration, suggesting that the decrease in expression is due to a reduction in the amount of NBC1 per cell. These images are typical of those seen in sections prepared from three control and three candesartan-treated rats. The results demonstrate that angiotensin II positively regulates NBC1 abundance via AT1 receptor occupation and could play a physiological role in regulation of proximal tubule acid–base transport.

Effects on NHE3

Total NHE3 abundance. Despite evidence for short-term effects of Ang-II on NaCl absorption from previous studies,[9] we saw no significant changes in the abundance of NHE3 in whole kidney homogenates (WKH) (Figure 3) or cortical homogenates (control: 100 ± 20; candesartan treated: 106 ± 16; blot not shown) in response to candesartan or Ang-II infusion. These results suggest that Ang II does not directly regulate total NHE3 abundance in rat kidney.

Another way of regulating NHE3 is through changes in the abundance of NHE3 in the apical brush border, which can occur without a change in total NHE3 through changes in the

Table 1 | Urinary excretion and serum composition

Variable	Candesartan[a]		Ang-II	
	Control	Experimental	Control	Experimental
Number	6	6	6	6
Urinary excretion (mmol/day)				
Na$^+$	0.3 ± 0.1	0.4 ± 0.1	2.5 ± 0.1	2.5 ± 0.1
K$^+$	1.0 ± 0.1	$0.8 \pm 0.1^*$	1.1 ± 0.1	1.1 ± 0.0
NH$_4^+$	0.97 ± 0.06	$0.73 \pm 0.07^*$	1.70 ± 0.10	2.03 ± 0.29
Serum				
Cl$^-$ (mEq/l)	100 ± 1	106 ± 2	100 ± 1	107 ± 1
HCO$_3^-$ (mmol/l)	26 ± 1	$20 \pm 1^*$	25 ± 0	$28 \pm 1^*$
Aldosterone (nmole/l)	1.0 ± 0.4	0.4 ± 0.2	0.1 ± 0.0	0.6 ± 0.2
Creatinine Cl (ml/min)	1.5 ± 0.1	1.2 ± 0.1	0.8 ± 0.0	$1.0^* \pm 0.1$

All values are mean $\pm$ s.e.m.
*$P < 0.05$.
[a]Values from the candesartan experiment (except for ammonium excretion values) were previously published[14] and are cited here for convenience.
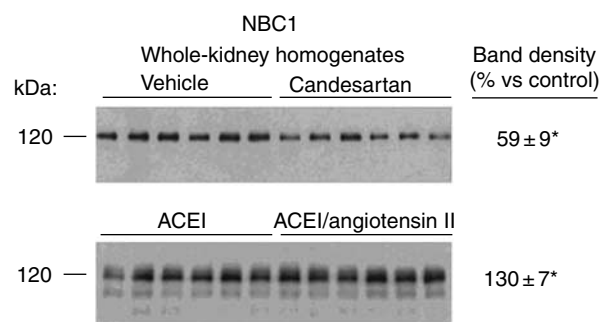

Figure 1 | NBC1 abundance in whole kidney homogenates. Effects of candesartan (1 mg/kg/day) blockade of the AT1 receptor administered to rats on low-sodium diet (0.5 mEq/200 g body weight/day) and Ang-II infusion (24 ng/min of Ang-II plus the ACE inhibitor lisinopril) in sodium replete rats. Results are from six control and six experimental animals in both experiments. The molecular weight of NBC1 is marked on the left of the figure. Values on the right were determined by densitometry expressed as percent of control abundance. *$P < 0.05$. Each lane of the immunoblots was loaded with a sample from a different rat. Candesartan infusion caused a significant decrease in NBC1 abundance, whereas Ang-II infusion resulted in a significant increase. ACEI = angiotensin-converting enzyme inhibitor.
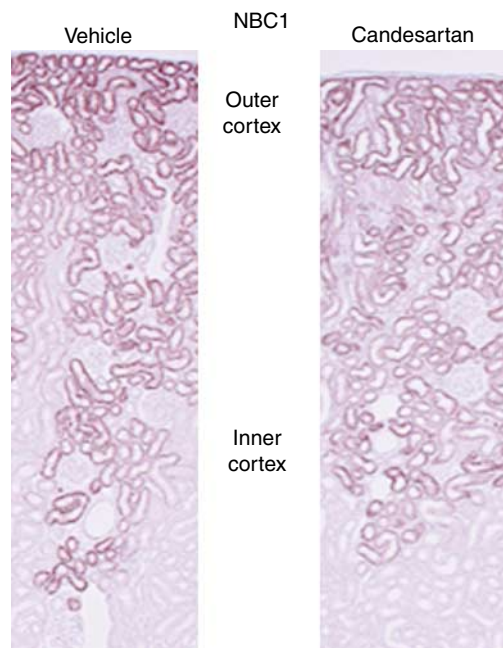
Figure 2 | Immunoperoxidase labeling of NBC1 in kidney sections (2 μ paraffin-embedded sections) from vehicle- and candesartan-treated rats. Each of the two sections shown are from different rats, one from a control and one from a candesartan-treated rat. The distribution of labeling of the basolateral membrane regions of proximal tubules did not change in response to candesartan treatment.

Figure 3 | NHE3 abundance in WKH and BBMs. *WKH:* Effects of candesartan (1 mg/kg per day) blockade of the AT1 receptor administered to rats on low-sodium diet (0.5 mEq/200 g body weight/day) and Ang-II infusion (24 ng/min of Ang-II plus the ACEI inhibitor lisinopril) in sodium replete rats. Results are from six control and six experimental animals in both experiments. Each lane of the immunoblots was loaded with a sample from a different rat. The molecular weight of NHE3 is marked on the left of the figure. Values on the right are abundance determined by densitometry expressed as percent of control abundance. *$P < 0.05$. Neither treatment resulted in significant differences from control abundance. *BBM fractions:* Effects of candesartan (1 mg/kg/day) blockade of the AT1 receptor administered to rats on low-sodium diet (0.5 mEq/200 g body weight/day) and Ang-II infusion (24 ng/min of Ang-II plus the ACE inhibitor lisinopril) in sodium replete rats. Results are from five control and five experimental animals in each study. NHE3 abundance decreased significantly in response to candesartan infusion ($P < 0.05$), but there was no change in response to Ang-II infusion. ACEI = angiotensin-converting enzyme inhibitor.

distribution of NHE3 among membrane subcompartments. To test that possibility, we measured NHE3 abundance in brush border membrane (BBM) fractions from candesartan-treated animals as well as from Ang-II-treated animals. Candesartan caused a small but significant decrease in BBM NHE3, while Ang-II did not change BBM NHE3 (Figure 3). Thus, Ang-II does not appear to cause a major change in the amount of NHE3 in the apical BBM, although it does not rule out changes in NHE3 within membrane subdomains contained in the brush border (see Discussion). Indeed, as shown in Figure 4 (NHE3 immunocytochemistry), there was no evidence of NHE3 internalization into the endosomal compartment in response to candesartan or Ang-II infusion.

Effects on NaPi-2

Another transporter in the apical plasma membrane of the proximal tubule is the sodium-phosphate cotransporter, NaPi-2. NaPi-2 is responsible for absorption of filtered phosphate, an important urinary buffer. As shown in Figure 5, although infusion of Ang-II had no significant effect on NaPi-2 abundance in WKH or BBM preparations, blockade of the AT1 receptor with candesartan caused a decrease in NaPi-2 abundance in WKH to $52 \pm 4\%$ of control levels. In BBM preparations, the reduction in NaPi-2 abundance to about 30% of control values, was even more marked (Figure 5). The large reduction in NaPi-2 abundance was also seen in immunostained sections (Figure 6), where the
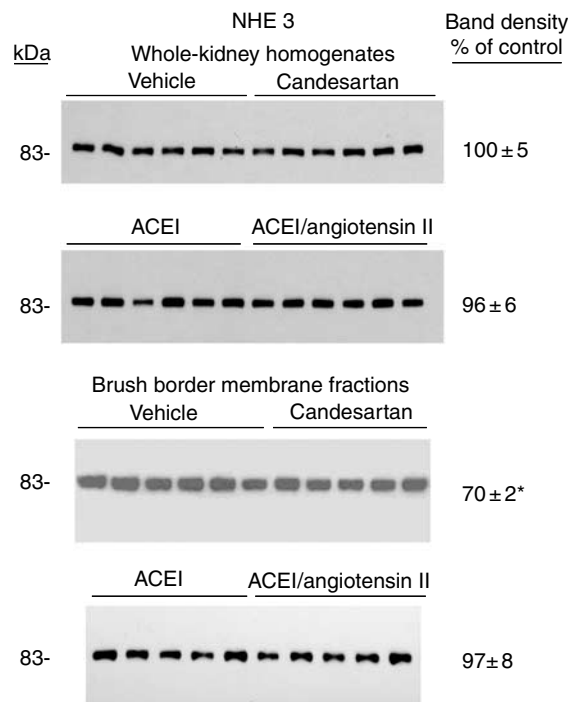
reduction in labeling of NaPi-2 in the inner cortex in sections from candesartan-treated rats was a consistent feature.

Effects on Na/K-ATPase

The Na/K-ATPase on the basolateral membrane maintains low intracellular sodium concentration, providing the driving force for a large number of secondary active transporters including NHE3 and NaPi-2. Recently, Yingst *et al.*[23] reported that Ang-II caused a rapid increase in Na/K-ATPase activity in isolated rat proximal tubules. However, neither candesartan nor Ang-II infusion significantly affected the abundance of the α-1 subunit of Na/K-ATPase (Figure 7).

NBC1, Na/K-ATPase, NHE3, and NaPi-2 mRNA abundances

To assess the effects of candesartan infusion on the transcript levels for the sodium transporters under investigation,
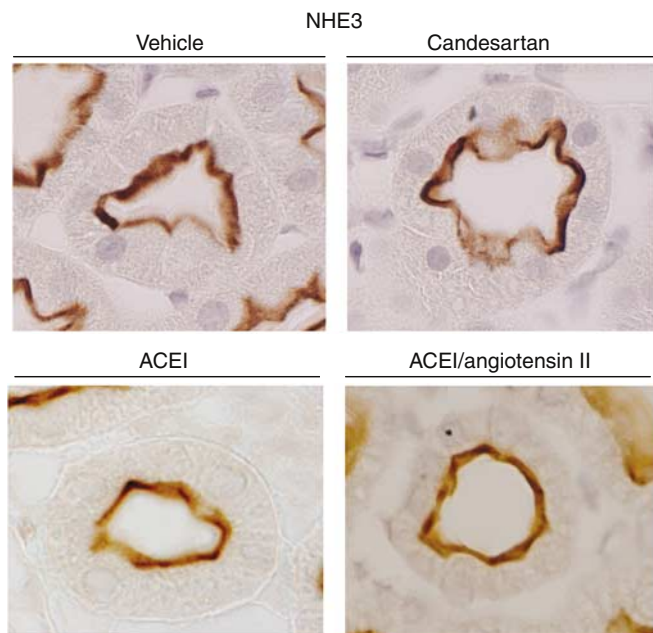
Figure 4 | Immunoperoxidase labeling of NHE3 in kidney sections (2 μ paraffin-embedded sections) from vehicle- and candesartan-treated rats, and from rats receiving lisinopril and vehicle versus lisinopril plus Ang-II. Labeling shows NHE3 concentrated in the brush border but no differences in NHE3 localization in response to candesartan or angiotensin II treatment are apparent. The sections are representative of three control and three experimental rats in each study.

Figure 5 | NaPi-2 abundance in WKH and BBMs. *WKH:* Effects of candesartan (1 mg/kg per day) blockade of the AT1 receptor administered to rats on low-sodium diet (0.5 mEq/200 g body weight/day) and Ang-II infusion (24 ng/min of Ang-II plus the ACE inhibitor lisinopril) in sodium replete rats. Results are from six control and six experimental animals in both experiments. Each lane of the immunoblots was loaded with a sample from a different rat. The molecular weight of NaPi-2 is marked on the left of the figure. Values on the right are abundances determined by densitometry expressed as percent of control abundance. *$P < 0.05$. Candesartan treatment caused a significant decrease compared to control abundance, but Ang-II infusion had no effect. *BBM fractions:* Effects of candesartan blockade of the AT1 receptor (1 mg/kg/day) administered to rats on low-sodium diet (0.5 mEq/200 g body weight/day) and Ang-II infusion (24 ng/min of Ang-II plus the ACE inhibitor lisinopril) in sodium replete rats. Results are from five control and five experimental animals. NaPi-2 abundance decreased significantly in response to candesartan infusion but there were no significant effects of Ang-II. ACEI = angiotensin-converting enzyme inhibitor.

real-time reverse transcriptase-polymerase chain reaction was performed using RNA samples from WKH. No significant differences in transcript levels were detected for any of the four transporters (Figure 8). This includes mRNA for NBC1 and NaPi-2 for which changes in protein abundance for the transporters were found. Candesartan treatment decreased NBC1 protein abundance by about 40% and NaPi-2 protein abundance by about 50% in WKH (Figures 1 and 5). These results suggest that a lower rate of translation or an increase in the rate of NBC1 and NaPi-2 degradation is responsible for the decreased abundances of NBC1 and NaPi-2 protein.

DISCUSSION

The results demonstrate that Ang-II can regulate the abundance of the basolateral bicarbonate ion transporter of the proximal tubule, NBC1, as well as the abundance of NaPi-2. These findings, along with those of earlier studies[1–5] showing effects of Ang-II on bicarbonate, proton, and ammonium transport, demonstrate the importance of Ang-II in regulation of proximal tubule acid–base transport.

Decreased receptor occupation owing to candesartan blockade and increased receptor occupation as a result of Ang-II infusion both altered acid–base balance. Plasma bicarbonate levels were reduced by candesartan but were increased in response to Ang-II infusion. In addition, candesartan treatment reduced urinary ammonium excretion.
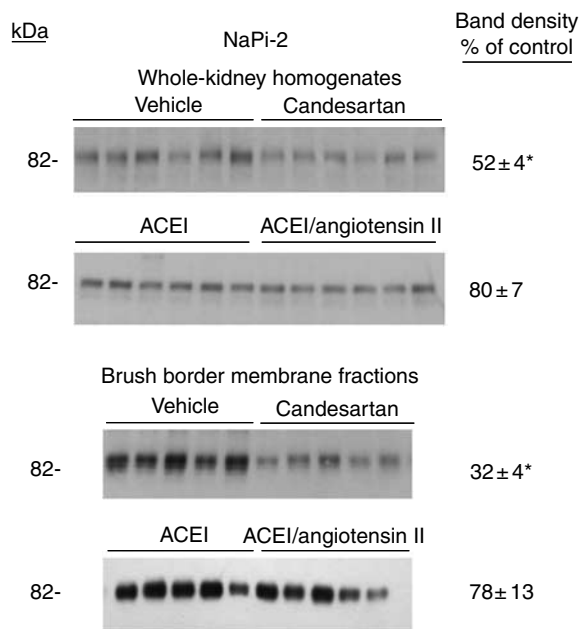
Conservation of plasma bicarbonate and excretion of ammonium in the proximal tubule are key components of the renal response to an acid load. Both these processes depend on production of bicarbonate and ammonium ions by glutamine metabolism in proximal tubule cells.[24] In addition, bicarbonate generated in proximal tubule cells by carbonic anhydrase-mediated hydroxyl ion hydration must be transported out of the cell across the basolateral membrane to affect the systemic acid–base state. Since NBC1 is the basolateral transporter by which most bicarbonate is delivered to the blood, a reduction in abundance, such as seen in these experiments in response to candesartan treatment, would be expected to impair bicarbonate conservation and acid–base homeostasis. Similarly, the increase in plasma bicarbonate observed in response to Ang-II infusion could have been mediated, in part, by the observed increased NBC1 abundance. The changes in NBC1 abundance in response to decreased or increased occupation
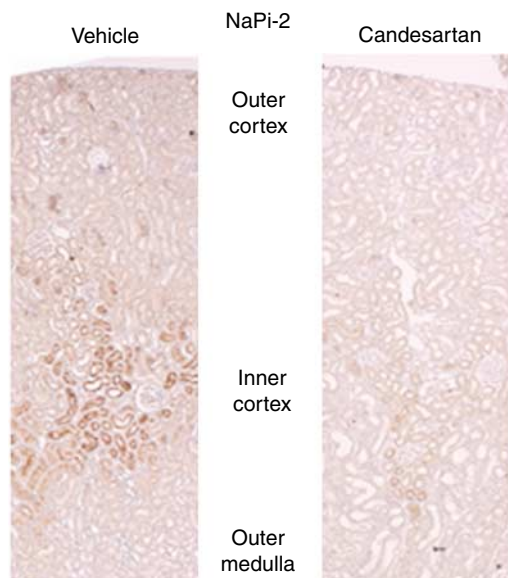
Figure 6 | Immunoperoxidase labeling of NaPi-2 in kidney sections (2 μ paraffin-embedded sections) from control and candesartan-treated (1 mg/kg/day) rats. Labeling shows heavy labeling of NaPi-2 in the inner cortex in sections from control rats. Labeling is decreased markedly in response to candesartan infusion. The sections are representative of three control and three candesartan-treated rats.
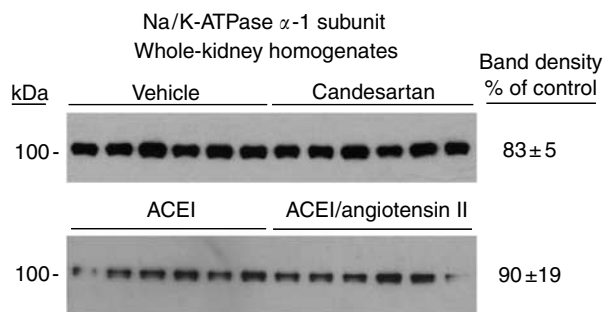

Figure 7 | Na/K-ATPase, α-1 subunit abundance in whole kidney homogenates. Effects of candesartan blockade of the AT1 receptor (1 mg/kg/day) administered to rats on low-sodium diet (0.5 mEq/200 g body weight/day) and Ang-II infusion (24 ng/min of Ang-II plus the ACE inhibitor lisinopril) in sodium replete rats. Results are from six control and six experimental animals in both experiments. The molecular weight of the α-1 subunit of Na/K-ATPase is marked on the left of the figure. Values on the right are abundances determined by densitometry expressed as percent of control abundance. Each lane of the immunoblots was loaded with a sample from a different rat. Neither treatment caused a significant change in abundance. ACEI = angiotensin-converting enzyme inhibitor.

Figure 8 | Effect of AT1 blockade on Na$^+$ transporter transcript levels. Transcript levels for the four sodium transporters studies in these experiments were determined by reverse transcriptase-polymerase chain reaction. Solid bars represent means of determinations from six rats in each group and the small vertical bars show s.e.m. values. Candesartan blockade of the AT1 receptor had no significant effect on mRNA amounts.

of the AT1 receptor by Ang-II seen in this study fit well with previous observations. Earlier work has shown that both sodium and bicarbonate absorption are stimulated by Ang-II[9,15,25] and that the response is mediated by Ang-II binding the AT1 receptor.[10,11]

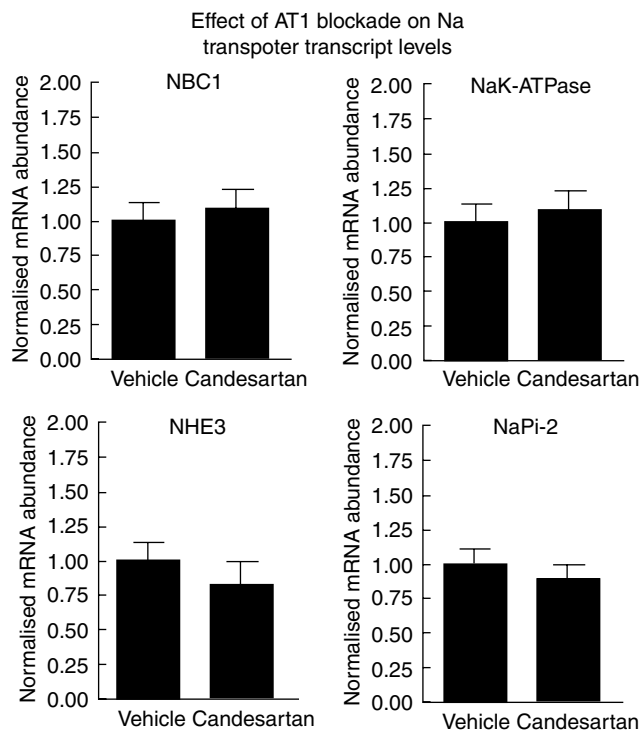The transporter whose abundance was most dramatically affected by candesartan treatment was NaPi-2. The decreases observed in WKH and BBM fractions could be in response to the apparent metabolic acidosis suggested by the low plasma bicarbonate ion levels seen in candesartan-treated rats. Previous studies in this laboratory demonstrated that NaPi-2 abundance decreases in response to acid loading.[19] In contrast to blockade of the AT1 receptor, Ang-II infusion did not significantly affect NaPi-2 abundance in either WKH or BBM fractions. This supports the hypothesis that the decrease in NaPi-2 abundance seen in response to candesartan was due to the apparent metabolic acidosis. Downregulation of NaPi-2 abundance would be expected to contribute to increased urinary phosphate excretion and thus to increased net acid excretion. In contrast, it is unlikely that the observed decrease in NBC1 abundance in response to candesartan was due to acidosis since Kwon et al.[26] found no change in NBC1 abundance in rats subjected to chronic metabolic acidosis.

In our studies, urinary ammonium excretion was reduced by candesartan to about 75% of control values due perhaps to the observed modest reduction of NHE3 abundance in BBM. Another, perhaps more likely, possibility is that impaired ammonium excretion in response to candesartan was a result of reduced ammonium production in proximal tubule cells. Nagami[5,27] demonstrated that ammonia production, as well as secretion, in the S2 segment of mouse
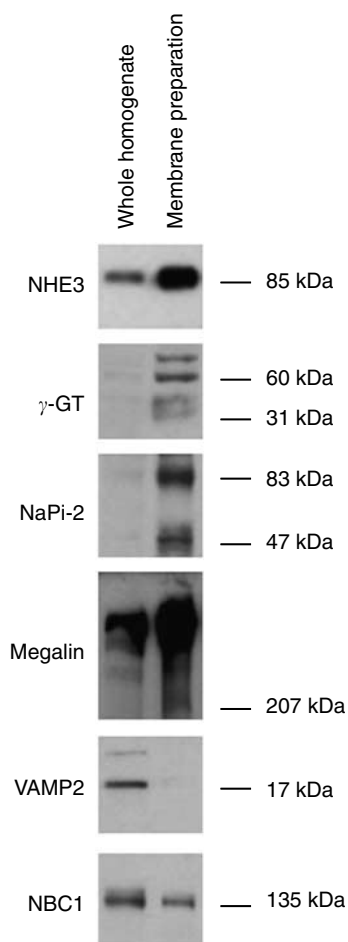
Figure 9 | Characterization of protein abundance from BBM preparations prepared by MgCl₂ precipitation from rats maintained on the low-sodium diet (0.5 mEq/200 g body weight/day). Molecular weights are marked for the predominant form of each transporter. Densitometric analysis showed that NHE3 was enriched to approximately 633% of the WKH amount. The apical membrane markers γ-glutamyltransferase and NaPi-2 were enriched about 10-fold and megalin was enriched about 3-fold, whereas the vesicle-associated membrane protein (VAMP2) concentration was decreased to about 10% of the WKH amount. NBC1 was decreased to about 57% of WKH.

proximal tubules is stimulated by Ang-II and that the effects are enhanced by acid loading.

Long-term candesartan treatment or angiotensin infusion had no effect on NHE3 abundance in kidney homogenates. Furthermore, there was little (with candesartan treatment) or no (with Ang-II infusion) change in BBM NHE3 with changes in AT1 receptor occupancy, a finding further supported by a lack of immunocytochemical evidence for NHE3 redistribution in the proximal tubule cell. This seemingly contrasts with the findings of other investigators, showing that physiological conditions that increase renal cortex NHE3 activity, such as chronic metabolic acidosis,[28,29] cause no change in NHE3 abundance in homogenates of the cortex[19] but can result in increased abundance of NHE3 in brush border.[30,31] However, the findings of Biemesderfer[32] and Yang *et al.*[33] appear compatible with the view that NHE3

is regulated in part through redistribution between different subregions of the apical plasma membrane, viz. the microvilli and the inter-microvillar clefts. Neither of our measurements (immunoblotting of BBM fractions or immunocytochemistry) would necessarily be expected to reveal such changes. Thus, we cannot rule out the possibility that the changes in AT1 receptor occupation in our studies were associated with such a microvillar to inter-microvillar transition. In addition to changes in abundance in the BBM, it is also possible that Ang-II regulates NHE3-mediated transport though post-translational modifications, induced protein–protein interactions, or altered ion gradients, mechanisms that would not be reflected in our immunoblots.

Candesartan treatment did not produce significant changes in the levels of mRNA for NBC1, NaPi-2, NHE3, or Na/K-ATPase. It is not unusual to see changes in transporter protein abundance without changes in message levels. Protein abundances are affected by their biological half-life as well as the half-life of the mRNA that codes for them.

CONCLUSION

Immunoblots and immunocytochemistry show that decreasing AT1 receptor occupation by candesartan blockade decreased abundance of NBC1, the principal bicarbonate transporter of the basolateral membrane in proximal tubules, whereas increased receptor occupation owing to Ang-II infusion increased NBC1 abundance. Changes in the activity of NBC1 would be expected to significantly affect systemic acid–base balance and in these experiments candesartan treatment of rats produced a 23% decrease in serum bicarbonate. Candesartan blockade of Ang-II binding to the AT1 receptor also caused significant decreases in the abundance of NaPi-2, a transporter in the apical membrane of proximal tubule cells that contributes to net acid excretion. These findings, interpreted in light of earlier studies, suggest that proximal tubule regulation of bicarbonate absorption by Ang-II is an important component of systemic acid–base homeostasis.

MATERIALS AND METHODS
Experimental animals and treatment protocols

Candesartan blockade of the AT1 receptor for Ang-II. To assess the effects of blockade of the AT1 receptor on transporter abundance, we infused candesartan into male Sprague–Dawley rats (224–250 g body weight) housed in metabolic cages under controlled temperature and light conditions (12 h day and night cycles). Sodium and water intake were controlled by ration feeding. Rats were given daily pre-weighed portions of an agar gel diet to provide (per 200 g body weight) 25 ml water and 15 g of nominally NaCl-free purified rodent chow (product #53140000, Zeigler Bros, Garners, PA, USA) with addition of 0.5 mEq NaCl/day.[34] This is a low level of NaCl intake, approximately 1/4th that of standard rat chow. Urine samples, collected daily, were preserved with thymol and evaporation was prevented with a layer of mineral oil. After a 4-day equilibration period on the low-sodium diet, rats were anesthetized with methoxyflurane (Metophane, Schering-Plough Animal Health

Corp., Union, NJ, USA) for subcutaneous implantation of osmotic minipumps containing candesartan (AstraZeneca, Mölndal, Sweden; a kind gift from Dr Peter Morsing). Candesartan was solubilized in $0.02 \, M \, Na_2CO_3$ in physiological saline. Experimental rats were given candesartan at a dose of 1 mg/kg/day, whereas control rats received only the saline/Na_2CO_3 vehicle.

The choice of a daily dietary sodium intake of 0.5 mEq/200 g body weight per day Na was based on preliminary studies which showed that lower intake of NaCl compromised renal function when combined with candesartan treatment. Specifically, in preliminary experiments, rats treated with candesartan and fed diets containing 0.03 or 0.3 mEq NaCl/200 g body weight showed evidence of decreased glomerular filtration rate in comparison to controls eating the low-salt diets but not treated with candesartan. At those lower salt levels, serum creatinine values were more than twice as high as control values and creatinine clearance was approximately 50% of control. In addition, immunoblots showed that the abundance of both NHE3 and Na/K-ATPase in cortical homogenates decreased significantly in the candesartan-treated groups (data not shown). At a dietary level of 0.5 mEq NaCl/200 g body weight, candesartan treatment did not significantly affect creatinine clearance or serum creatinine levels (Table 1).

After 2 days, animals were killed and trunk blood was collected. For semiquantitative immunoblotting, kidneys were rapidly removed and processed. For immunocytochemistry, animals were anesthetized as above and kidneys were perfusion-fixed in situ. Perfusion pressure was controlled and did not exceed 100 mm Hg.

Ang-II infusion

To assess the effects of increased occupation of the AT1 receptor by Ang-II on transporter abundance, we infused Ang-II into male Sprague–Dawley rats (224–250 g body weight) housed in metabolic cages and ration-fed the same amounts of water and food as given in the candesartan-infusion experiments, except that in these experiments 2.0 mEq NaCl/200 g body weight was added to the food ration. This is a standard level of salt intake for rats. Urine samples were collected as above. All rats in this experiment received long-term infusions of the angiotensin-converting enzyme inhibitor, lisinopril (AstraZenica; a kind gift from Dr Peter Morsing), and either Ang-II (Calbiochem, LaJolla, CA, USA) or the vehicle for Ang-II, described as follows. After 1 day of acclimation in metabolic cages, rats were anesthetized with isoflurane (Abbott Laboratories, North Chicago, IL, USA) and osmotic minipumps (Alzet, Cupertino, CA, USA) were implanted subcutaneously. The lisinopril (3 mg/kg/day) was given to all rats to minimize the endogenous production of Ang-II. Separate minipumps delivered 24 ng/min of Ang-II dissolved in 0.001 N acetic acid to experimental animals or vehicle (0.001 N acetic acid) alone to control rats. This was a mid-dose level from a previous study in our laboratory[14] and was within the range that resulted in increases in α-epithelial Na channel protein abundance. The dose used was designed to be a so-called 'non-pressor' dose, meaning that the dose is low relative to that needed for an immediate increase in blood pressure. After 3 days of treatment, rats were killed by decapitation, and kidneys were rapidly removed and prepared for semiquantitative immunoblotting. For immunocytochemistry, animals were anesthetized as above and kidneys were perfusion-fixed in situ.

All experiments were carried out in accordance with the Guide for Care and Use of Laboratory Animals and with the approval of the Institutional Animal Care and Use Committee.

Urine and serum chemistry

Measurements of urine and serum chemistry were done using an autoanalyzer (Monarch 2000 autoanalyzer, Instrumentation Laboratories, Lexington, MA, USA). Urine ammonium was determined using an enzymatic method (Sigma Diagnostics Procedure No. 170-UV). Serum aldosterone concentration (Coat-A-Count Aldosterone, Diagnostic Products Corp, Los Angeles, CA, USA) was measured by radioimmunoassay of serum.

Semiquantitative immunoblotting

The methods used have been described in detail elsewhere.[35,36] In all experiments, Coomassie-stained 'loading-control' gels were prepared to ensure equality of loading. To do this, sodium dodecyl sulfate -solubilized (solubilized with Laemmli sample buffer) samples of each homogenate or isolated membrane preparation were run on 12% polyacrylamide/sodium dodecyl sulfate gels. Selected bands on the loading gels were quantified by densitometry. Loading of gels for immunoblotting was adjusted according to the densitometry results to assure equal loading within 5% of the mean.

Antibodies

The antibodies used for immunoblotting and immunocytochemistry have been characterized by Kim et al.[19,35] (NHE3 and NaPi-2) and Schmitt et al.[37] (NBC1). A mouse monoclonal antibody against the α-subunit of Na^+/K^+-ATPase was obtained from Upstate Biotechnology (Lake Placid, NY, USA).

Membrane fraction preparation

Kidneys of rats were rapidly removed and chilled in ice-cold PBS. Aliquots of whole kidney homogenate (WKH) from each rat were used to prepare membrane fractions by the Mg-precipitation method as described by Biber et al.[38] and Fernandez-Llama et al.[39] The pellet resulting from the final centrifugation, consisting largely of apical plasma membranes of renal tubule epithelial cells, was re-suspended in isolation solution and solubilized in Laemmli sample buffer for subsequent immunoblotting.

Composition of the final pellet was determined by comparing membrane fraction isolate with WKH from the same rat. Previous characterization by Biber et al.[38] showed that the final pellet was enriched in marker enzymes of the brush border (alkaline phosphatase and aminopeptidase M). Marker enzymes for mitochondria, endoplasmic reticulum, and secretory vesicles were absent. We characterized our membrane fractions by immunoblotting (Figure 9) using antibodies to the brush border enzyme γ-glutamyltransferase (a kind gift of Dr Rebecca Hughey, University of Pittsburgh),[40] vesicle-associated membrane protein,[34] NaPi-2,[19] and NBC1 (Chemicon International Inc., Temecula, CA, USA). Immunoblots showed appropriate enrichment of BBM marker proteins. Specifically, the apical membrane markers γ-glutamyltransferase and NaPi-2 were enriched about 10-fold and megalin was enriched nearly 3-fold. NHE3 was enriched about 6-fold, whereas the vesicle-associated membrane protein abundance was decreased to about 10% of the WKH amount. The basolateral membrane protein NBC1 was de-enriched to about 57% of WKH levels.

Immunocytochemistry

Kidneys were perfusion-fixed with a paraformaldehyde-based fixative and $2 \, \mu$ paraffin-embedded sections were prepared as described by Nielsen et al.[41] Sections were labeled using the immunoperoxidase method of Hager et al.[42]

Real-time reverse transcriptase-polymerase chain reaction

Quantitative, real-time reverse transcription-polymerase chain reaction (ABI Prism 7900HT) was used to measure relative mRNA abundances in kidneys of vehicle-treated and candesartan-treated rats as previously described.[43] The primer sequences used are given as supplementary materials in Brooks et al.[43] Relative quantification of gene expression was achieved using the comparative C_T method. Validation experiments were performed to show that amplification efficiencies were equal between control and experimental groups.[44] Specificity of the amplified products was determined using melting curve analysis and by product sequencing.

Statistical analysis

Values for experimental rats were compared with controls using an unpaired t-test when s.ds. were the same or by Welch's t-test when s.ds. were significantly different (INSTAT; Graphpad Software, San Diego, CA, USA). Relative quantification of the band densities from immunoblots was carried out by densitometry using a laser densitometer (Molecular Dynamics, San Jose, CA, USA) and ImageQuaNT software (Molecular Dynamics). To facilitate comparisons, we normalized the densitometry values such that the mean for the control group is defined as 100%. $P < 0.05$ was considered statistically significant. All numerical values are reported as mean $\pm$ s.e.m.

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