Proceedings of the Australian Physiological and Pharmacological Society (2004) ***34:*** *71-74* *http://www.apps.org.au/Proceedings/34/71-74*

Activation of renal calcium and water excretion by novel physiological and pharmacological activators of the calcium-sensing receptor

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Summary

1. Activated Ca^{2+}-sensing receptors (CaRs) play key roles in the regulation of whole body calcium metabolism by inhibiting the secretion of the key calcitropic hormone, PTH and promoting urinary calcium excretion.

2. We have now examined the effects of intravenous administration of novel calcium receptor activators on renal function in anaesthetized female Wistar rats.

3. The type-II calcimimetic, NPS R467 and the CaR-active amino acids, L-Phe and L-Ala, which act at distinct binding sites on the receptor all activated urinary flow rate, calcium and osmolar excretion and suppressed urinary osmolality.

4. The effects of L-Phe and NPS R-467 on urine flow rate and calcium excretion were stereoselective consistent with the idea that these effects were mediated by calcium-sensing receptors.

5. However, D-Phe also suppressed urinary osmolality and promoted osmolar excretion possibly by exceeding the transport maximum in the proximal tubule.

6. The data indicate that novel activators of calcium-sensing receptors, including L-amino acids at physiologically relevant serum concentrations, play a significant role in the regulation of urinary calcium and water excretion.

Introduction

The calcium-sensing receptor (CaR) is a member of group C of the G-protein coupled receptor super-family. These receptors play multiple roles in calcium homeostasis including key roles in mediating the feedback regulation of parathyroid hormone secretion and urinary calcium excretion. Inactivating mutations of the CaR underlie several human pathological states including the relatively benign condition familial hypocalciuric hypercalcemia and its more severe but much rarer homozygous form, neonatal severe hyperparathyroidism which requires parathyroidectomy within the first few weeks of life (review:[1]). The widespread distribution of these receptors, together with their resistance to desensitization, points to much wider roles in mammalian physiology (review:[2]).

Recently, two new classes of calcium-sensing receptor (CaR) activators have been identified. The type-II calcimimetics (e.g., NPS R-467 and R-568) were developed from a lead phenylalkylamine compound identified in a large-scale drug screen[3]. Type-II calcimimetics sensitize the CaR to calcium ions by binding to a site in the receptor's transmembrane region[4]. More recently, several sub-classes of L-amino acids (including aromatic, polar, and aliphatic amino acids) have been shown to act as allosteric activators of the CaR. Furthermore, physiologically relevant fluctuations in the concentration of physiological amino acid mixtures can modulate receptor activity[5]. The amino acid binding site is likely to lie in the conserved N-terminal, Venus FlyTrap domain[6].

In the kidney, the CaR is expressed in multiple sites. These include the apical membrane of the proximal tubule, the basolateral membrane of the cortical thick ascending limb (CTAL) and the apical membrane of the medullary collecting ducts (review:[7]). Thus, fluctuations in the serum levels of Ca^{2+} and amino acids might be expected to modulate CaR activity in the CTAL and fluctuations in the tubular fluid levels of Ca^{2+} and amino acids might be expected to modulate CaR activity in the proximal tubule and collecting ducts. Consistent with a role for the CaR in the regulation of urinary phosphate excretion, dietary phosphate loading has been shown to suppress the expression of the CaR in the apical brush border membrane of the proximal tubule[8]. Furthermore, the CaR agonist gadolinium (Gd^{3+}) and the type-II calcimimetic NPS R-467 reversed PTH suppression of phosphate reabsorption in cultured proximal tubule cells[9]. On the other hand, expression of the CaR in the CTAL has been linked to the control of urinary calcium excretion and expression of the CaR in the collecting tubule has been linked to the control of urinary water excretion and osmolality. In particular, CaR activation suppresses vasopressin-induced water reabsorption, facilitating the excretion of solutes such as calcium, phosphate and oxalate that might otherwise contribute to the formation of renal calculi[10].

The patterns of expression described above imply roles for CaR activators in the regulation of multiple renal functions including proximal tubular transport, calcium excretion and urinary concentration. For example, CaR-active amino acids (e.g., L-Phe and L-Ala) and type-II calcimimetics are predicted to promote calcium excretion (Fig. 1) and raise urine flow and suppress urinary osmolality (Fig. 2). We have examined the impact of intravenously administered L-amino acids or the type-II calcimimetic, NPS R-467 on renal calcium and water excretion in rats and report herein our preliminary findings. The data provide support for the hypotheses that CaR activators including L-amino acids promote urinary calcium and water excretion.

Materials and Methods

NPS R-467 and its 100-fold less potent isomer S-467 were the generous gifts of Dr Edward Nemeth (NPS Pharmaceuticals, Toronto, Canada). Animal experiments on a total of approximately forty rats were performed with approval from the University of Sydney Animal Ethics

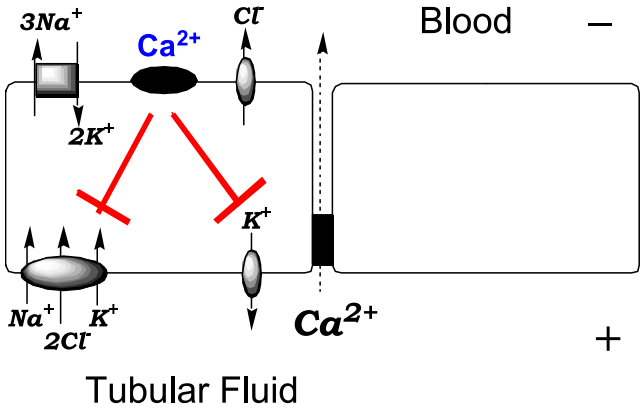

Figure 1. Schematic diagram of a thick ascending limb cell. *The diagram shows the inhibitory effect of calcium-sensing receptor activation on apical $Na^+/K^+/2Cl^-$ co-transport and K^+ recycling. The impact of CaR activation is believed to be a reduction in the lumen-positive potential difference that drives Ca^{2+} reabsorption.*

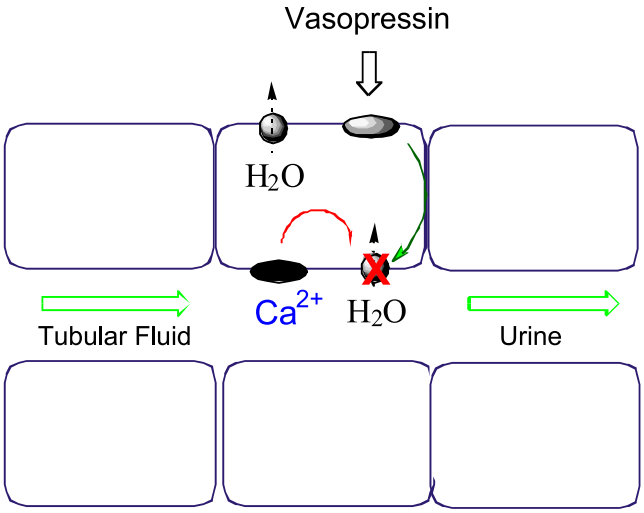

Figure 2. Schematic diagram of an epithelial cell from the collecting tubules. *The diagram shows the inhibitory effect of CaR activation on vasopressin stimulated water reabsorption via aquaporin-2. In this way, CaR activators that have entered the renal filtrate and have not been reabsorbed in the proximal nephron may promote urinary water excretion.*

Committee. Female Wistar rats (200-300 g) were anaesthetized with halothane (2% in oxygen; 0.8 mL/min) then catheterized. Both jugular veins were cannulated and the animals were infused at a constant rate (4 or 5 mL per h) with an isotonic physiological saline solution of the following composition: 140 mM NaCl, 4.0 mM KCl, 15 mM $NaHCO_3$, 2.5 mM $CaCl_2$, 1 mM $MgCl_2$. After a 60 min equilibration period, continuous infusions of D and/or L-amino acids were commenced, continuing for 60 min prior to return to the control solution. Blood samples (0.25 mL) were collected at regular intervals for analysis of serum creatinine, osmolality, total calcium and various amino acids. Urine samples were collected at 15 min intervals to assess flow rate, osmolality and the concentrations of creatinine, calcium and amino acids. Osmolality was determined by vapour pressure osmometry. Creatinine was determined by an adaptation of the alkaline picrate method[11] using a Wallac Victor2 multi-well plate reader and serum and urine total calcium concentrations were determined using an autoanalyzer (Roche/Hitachi 912). Amino acid levels in serum and urine were determined by HPLC separation and fluorimetric detection of O-phthaldialdehyde-conjugates[12]. In some experiments, bolus injections were administered to test for acute effects of R-467, S-467 and amino acids including L-Phe and L-Ala. The data are routinely expressed as means ± SEM (number of experiments).

Results and Discussion

The type-II calcimimetic R-467 administered as a bolus intravenous injection of 2 µmol stereoselectively enhanced urinary calcium excretion (by 3-4 fold; Fig. 3A) and also promoted urinary flow rate (Fig. 3B). In addition, R-467 stereoselectively suppressed urinary osmolality from a baseline level of 946 ± 70 mosm/kg to 723 ± 80 mosm/kg (n = 4; p = 0.01) after 15 min consistent with an inhibitory action of the CaR on vasopressin-induced water reabsorption in the collecting ducts. Although the osmolality dropped, the osmolar excretion rate increased following exposure to R-467. The baseline osmolar excretion rate was 12.5 ± 2.4 µosmol/min and this increased to 34.6 ± 0.8 µosmol/min following R-467 (n = 3; p = 0.01). R-467 also lowered serum total calcium levels (not shown) as previously reported for the related calcimimetic R-568[13]. The 100-fold less potent isomer, S-467 was much less effective than R-467 on all of the parameters tested.

Infusions of the CaR-active L-amino acid, L-Phe sufficient to raise the serum level from 0.05 mM to about 2 mM (determined by HPLC), also elevated urinary calcium excretion (by about 2-fold; Fig. 4A) and urinary flow rate (Fig. 4B). Both effects were L/D selective (Fig. 4). In addition, both D-Phe and L-Phe reversibly suppressed urinary osmolality. In the case of L-Phe, urinary osmolality was maximally suppressed from 767 ± 20 mosm/kg to 599 ± 17 mosm/kg (n = 3) after 60 min. The reason for the apparent lack of L/D selectivity of this effect is not clear. However, it may have arisen from higher local D-amino acid concentrations in the tubular fluid as a result of the selectivity of proximal tubular amino acid transporters for L-amino acids. In addition, the osmolar excretion rate was significantly increased following exposure to L-Phe and D-Phe. The baseline osmolar excretion rate was 13.2 ± 3.6 µosmol/min and this increased to 33.4 ± 4.2 µosmol/min following D-Phe (n = 3; p < 0.01) and 36.7 ± 5.3 µosmol/min following L-Phe (n = 3; p < 0.01). Bolus injections of L-Phe and L-Ala also acutely elevated urinary

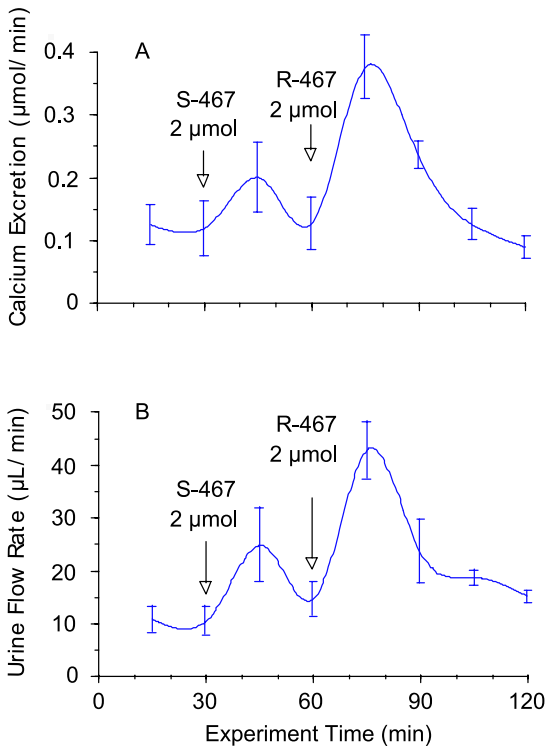

Figure 3. Effects of NPS R-467 on urinary calcium excretion and flow rate. *Female Wistar rats were anaesthetised and infused intravenously with physiological saline at a rate of 4 mL/h via a jugular vein cannula. The CaR activator, R-467 and its stereoisomer S-467 were delivered intravenously as bolus injections (0.5 mL) in physiological saline. The data are means ± SEM (n=4).*

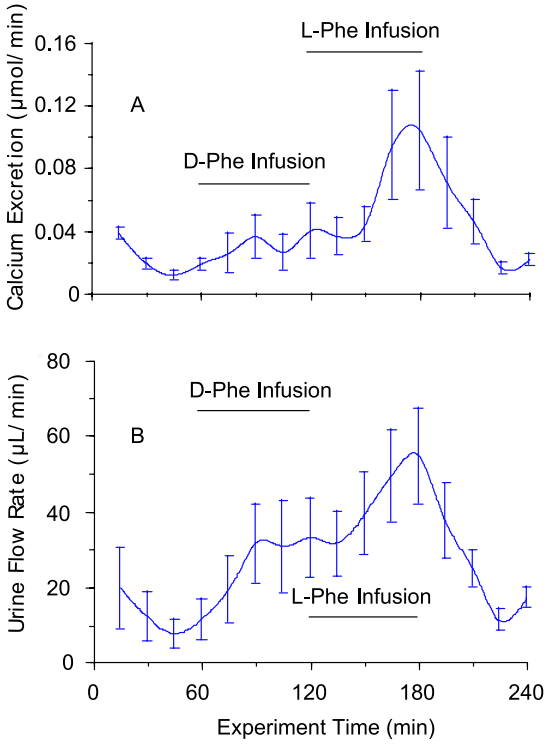

Figure 4. Effects of L-Phe and D-Phe infusions on urinary calcium excretion and flow rate. *Female Wistar rats were anaesthetised and infused intravenously with physiological saline at a rate of 4 mL/h via a jugular vein cannula. After 60 min, the infusion was switched to saline that contained D-Phe (200 mM) and, after 120 min, to saline that contained L-Phe (200 mM). The maximum plasma amino acid concentration observed under the conditions of these experiments was approximately 2 mM (baseline level around 0.05 mM) and the urine amino acid concentration rose to around 20 mM in the case of D-Phe and around 10 mM in the case of L-Phe. The data are means ± SEM (n=4).*

calcium excretion and flow rate and lowered urinary osmolality (not shown).

Taken together the data are consistent with the idea that novel activators of the CaR including L-amino acids and type-II calcimimetics such as R-467 mimic the effects of elevated plasma Ca^{2+} concentration on urinary calcium excretion, flow rate and osmolality. Increased serum and urinary amino acid concentrations associated, for example, with elevated dietary protein intake appear to act as physiological signals for enhanced solute and water excretion. CaR activators from distinct structural groups and targeting distinct binding sites on the receptor have similar effects on renal calcium and water excretion consistent with the proposed sites of CaR action in the thick ascending limb and collecting tubules.

Acknowledgements

The author's work is supported by the NHMRC of Australia.

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Received 30 October 2003; revised 27 January 2004. Accepted 29 January 2004.

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Molecular changes in proximal tubule function in diabetes mellitus

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Summary

1. Diabetic kidney disease is initially associated with hypertension and increased urinary albumin excretion. The hypertension is mediated by enhanced volume expansion due to enhanced salt and water retention by the kidney. The increased urinary albumin is not only due to increased glomerular leak but to a decrease in albumin reabsorption by the proximal tubule. The precise molecular mechanisms underlying these two phenomena and whether there is any link between the increase Na^+ retention and proteinuria remain unresolved.

2. There is significant evidence to suggest that increased Na^+ retention by the proximal tubule Na^+-H^+ exchange isoform 3 (NHE3) can play a role in some forms of hypertension. Increased NHE3 activity in models of diabetes mellitus, may explain in part the enhanced salt retention observed in patients with diabetic kidney disease.

3. NHE3 also plays a role in receptor mediated albumin uptake in the proximal tubule. The uptake of albumin requires the assembly of a macromolecular complex that is thought to include the megalin/cubulin receptor, NHE3, the vacuolar type H^+-ATPase (v-H^+-ATPase), the Cl^- channel, ClC-5 and interactions with the actin cytoskeleton. NHE3 seems to exist in two functionally distinct membrane domains, one involved with Na^+ reabsorption and the other involved in albumin uptake.

4. This review focuses on the evidence derived from in vivo studies as well as complementary studies in cell culture models for a dual role of NHE3 in both Na^+ retention and albumin uptake. We suggest a possible mechanism by which disruption of the proximal tubule albumin uptake mechanism in diabetes mellitus may lead to both increased Na^+ retention and proteinuria.

Diabetes mellitus, hypertension and albuminuria

Diabetic nephropathy is the most prevalent cause of chronic renal failure and end-stage renal disease in the Western world and can account for up to 40% of the patients requiring renal replacement therapy[1]. The onset of renal failure in patients with diabetes mellitus is associated with hypertension and increased urinary albumin excretion[2]. Although mesangial expansion, glomerular hypertrophy and thickening of the glomerular basement membrane leading to hyperfiltration and microalbuminuria are hallmarks of diabetic nephropathy, it is the degree of interstitial fibrosis that more closely correlates with the decline in glomerular filtration rate[3]. The tubulointerstitium represents a dynamic environment that maintains the structural and functional homeostasis within the kidney and it is the dysregulation of this highly integrated system that may lead to many of the complications associated with diabetic kidney disease[4].

The hypertension usually observed in patients with diabetic nephropathy is well recognised to be mediated by volume expansion due to enhanced salt and water retention by the kidney[5]. This suggests a dysregulation of the normal mechanisms to maintain volume homeostasis occurs in the 'diabetic milieu' long before a functional decline in renal function develops. Microalbuminuria is well recognised as being associated with primary glomerular pathology[6]. However, there is now clear evidence that the renal tubule has a critical role in the reabsorption of filtered albumin and in the development of albuminuria[7]. As microalbuminuria and volume-mediated hypertension occur in patients with diabetes mellitus, this may suggest a more direct relationship between albumin handling and Na^+ reabsorption. This review will focus on the possible compartmentalised roles of NHE3 in Na^+ reabsorption and albumin uptake in the proximal tubule and how the trafficking of NHE3 between the two functional compartments may provide a link to explain the co-existence of hypertension and albuminuria in diabetic nephropathy.

Under normal conditions, the kidneys filter approximately 180 litres of blood and reabsorb approximately 1.7 kg of NaCl per day[8]. The proximal tubule facilitates 'bulk' reabsorption of Na^+, responsible for 50-75% of tubular Na^+ reabsorption. At the brush border membrane of proximal tubules approximately 0.7 moles of sodium are reabsorbed per hour [9]. Thus relatively small changes in the capacity of the proximal tubule to reabsorb Na^+ and water in response to elevations in plasma glucose or cytokine levels may result in dramatic changes in Na^+ retention and volume expansion.

Na^+-H^+ Exchanger Isoform 3 and Na^+ Retention

The luminal reabsorption of Na^+ in the proximal tubule is achieved primarily by the secondary active transport of the Na^+-H^+ exchanger isoform 3 (NHE3) mediated by the Na^+ gradient generated by the basolateral Na^+-K^+-ATPase[8]. There are now several lines of evidence to suggest that changes in the activity of NHE3 may be linked to hypertension. Importantly, a recent study in hypertensive patients found that proximal tubule Na^+ reabsorption was an independent determinant of the blood pressure in volume-dependent hypertension[10]. Similarly, a reduction in NHE3 activity has been reported in acute hypertension[11,12] implicating a role for NHE3 in pressure natriuresis.

Several studies in spontaneously hypertensive rats (SHR), a commonly used model for human essential hypertension, are consistent with a role for NHE3 in the genesis of volume expansion. In the tubules of normal rats, Na^+-H^+ exchange activity was inhibited by parathyroid hormone (PTH) and dopamine but stimulated by angiotensin II (AngII) and norepinephrine. Tubules obtained from SHR tubules, however, were not responsive to PTH or dopamine and the levels of stimulation by AngII and norepinephrine were significantly reduced[13]. These imbalances could contribute to the development and maintenance of hypertension in this model[13]. NHE3 activity was also found to be elevated in a further study in SHR rats, with v-H^+-ATPase also implicated in the regulation of Na^+ transport in the proximal tubule[14]. Consistent with the above studies, proximal tubule cells freshly isolated from SHR demonstrate a 3-fold increase in NHE3 activity with a 50% increase in NHE3 protein[15]. Furthermore, in SHR there appears to be defective coupling of the dopamine receptor to adenyl cyclase, resulting in an alleviation of the cAMP mediated inhibition of NHE3, with subsequent elevation in Na^+ retention[16]. In a more recent study, it was found that proximal tubules of 5 week old SHR had greater levels of NHE3 and v-H^+-ATPase activity compared to age matched normotensive Donryu rats. These findings led the authors to conclude that enhanced proximal tubule fluid reabsorption is likely to contribute to the development of high blood pressure in young SHR[17]. Immunofluorescence studies revealed that there was a significant level of redistribution of NHE3 in the proximal tubules in both SHR and Goldblatt hypertensive rats providing evidence for the dynamic role of NHE3 in states known to alter proximal tubular Na^+ reabsorption[12].

Further conclusive evidence for the role of NHE3 in control of blood pressure was demonstrated using NHE3 knockout transgenic mice. Microperfusion studies revealed that fluid and HCO_3^- reabsorption were reduced by ~60-70%, demonstrating that NHE3 is the major apical transporter mediating Na^+ and HCO_3^- reabsorption in the proximal tubule. These changes were associated with small but significant decreases in blood pH and HCO_3^- [18]. Importantly, the systolic and mean arterial blood pressures in these mice were significantly reduced. These data therefore support the view that the major renal Na^+ transporters, including NHE3, play a central role in long-term control of arterial blood pressure[19,20].

NHE3 in diabetes mellitus

As discussed above, diabetes mellitus is associated with renal NaCl retention and expanded extracellular fluid volume, characterised by systemic suppression of the renin-angiotensin system. Volume expansion is largely responsible for hypertension in diabetes mellitus and may contribute to the altered haemodynamics responsible for diabetic nephropathy. Diabetes mellitus is associated with chronic or intermittently high plasma glucose levels, which are implicated in a number of adverse effects on the kidney. There is evidence to suggest that increased Na^+ flux with glucose via SGLT-1 transporters may contribute to increased Na^+ reabsorption by the kidney[21]. However, when the significant role that NHE3 plays in Na^+ and fluid reabsorption in the proximal tubule is taken into account, hyperglycaemia-induced increases in the activity of NHE3 potentially also contribute to increased Na^+ retention and related volume expansion.

The first evidence that NHE3 was increased in the proximal tubule in diabetes was provided by Harris and co-workers in 1986[22] who demonstrated increased Na^+-H^+ exchange in brush border vesicles from rats induced to diabetes with streptozocin (STZ). Micropuncture studies in our own laboratories have also clearly demonstrated in STZ rats that there is a pronounced increase in tubular Na^+ reabsorption[23,24] and that this increase was primarily due to enhanced NHE3 activity[25]. *In vivo* models of diabetes mellitus using STZ rats have also demonstrated altered renal handing of H^+ and increased HCO_3^- absorption, a result attributable to increased NHE3 activity[26]. *In vitro* analysis of intact tubules and freshly isolated proximal tubule cells from STZ rats has shown increased NHE3 protein expression and activity[27]. In addition, studies from our lab and others in cultured opossum kidney (OK) cells have shown that exposure to high glucose for 48 hours results in a significant increase in both NHE3 mRNA and protein[28,29].

Furthermore, there have been at least two reports in humans that show increased proximal tubular Na^+ reabsorption in patients with diabetes mellitus. A study in children with Type 1 diabetes found a significant increase (~20%) in proximal tubular reabsorption as determined by fractional lithium clearance[30]. Similar studies in adults with Type 2 diabetes also found a ~20% change in reabsorption rates[31]. Thus, considerable evidence exists that the NHE3 mediated component of renal salt reabsorption may be at least in part responsible for the hypertension observed in patients with diabetes mellitus.

Albumin uptake in the proximal tubule - a macromolecular complex reliant on NHE3 activity?

It has long been recognised that the proximal tubule has a crucial role in reabsorbing any filtered albumin[32]. The concentration of albumin in the glomerular filtrate in rats and dogs ranges from <1 to 50 mg/l[32]. Recently, the concentration of albumin in humans has been estimated to be 3.5 mg/l[33] which translates to approximately 630 mg of albumin being filtered per day by the human kidneys. However, only around 30 mg is normally excreted in the urine per day, indicating that the tubules reabsorb at least 95% of all albumin filtered at the glomerulus. The uptake of albumin by the proximal tubule from the glomerular filtrate has been shown to occur by a highly active receptor-mediated endocytotic pathway involving the megalin/cubulin complex[34] (Figure 1). The albumin is then trafficked to the lysosomes where it is broken down to its constituent amino acids[34]. Importantly, the C-terminus of megalin contains numerous potential protein binding domains[35]. Recently it has been demonstrated that efficient

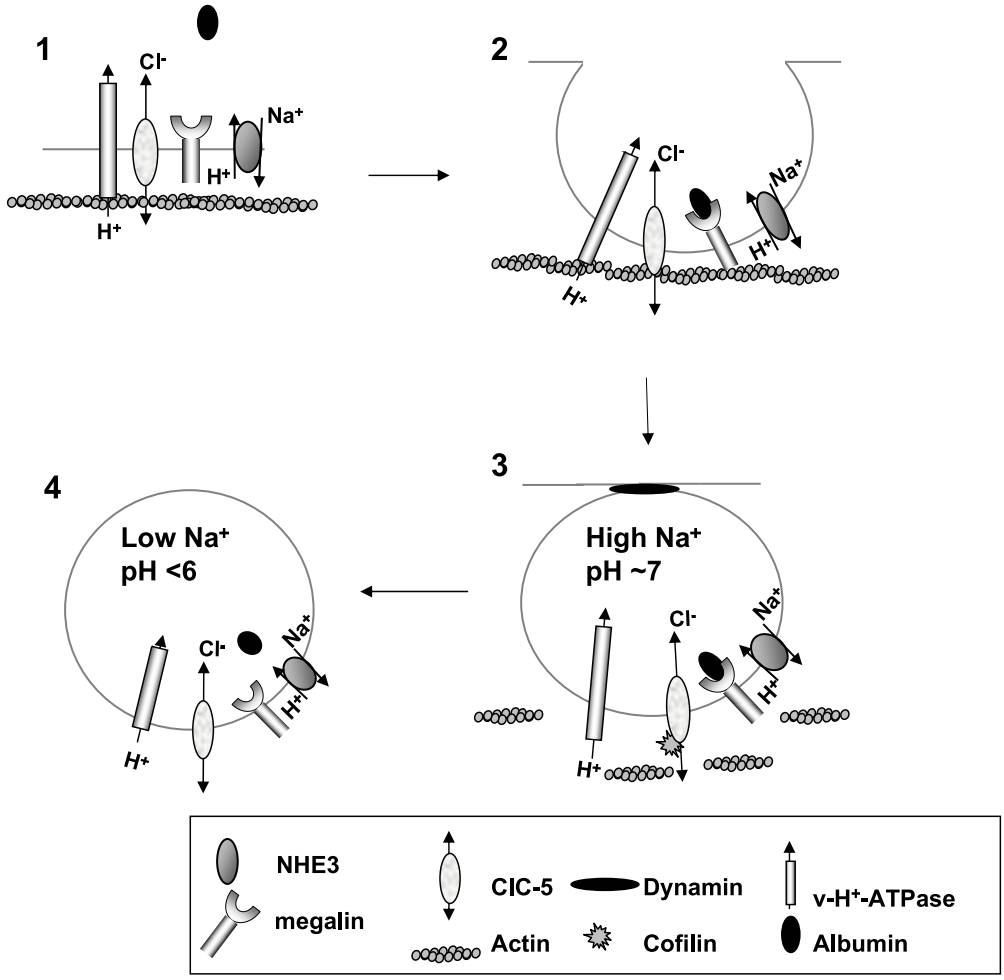

Figure 1. Macromolecular complex involved in proximal tubule albumin endocytosis. *(1) In the plasma membrane at the intravillar cleft ClC-5, v-H^+-ATPase, NHE3 and megalin associate by C-terminal tail interactions with scaffold proteins that anchor the complex to the actin cytoskeleton. (2) When albumin binds the megalin/cubulin complex, endocytosis is initiated. (3) As the nascent endosome forms it is pinched off from the membrane by dynamin. Entry into the cytoplasm requires the dissolution of the local actin filaments. This involves the C-terminal tail of ClC-5 recruiting the actin depolymerising protein cofilin to the complex. At this stage the endosome contains extracellular fluid high in Na^+ with a neutral pH. It is thought that NHE3 may initiate endosomal acidification by electroneutral exchange of endosomal Na^+ for cytosolic H^+. (4) When the Na^+ gradient is dissipated, the v-H^+-ATPase continues the acidification and ClC-5 provides the necessary anion shunt and albumin dissociates from the megalin/cubulin complex.*

trafficking of megalin through the endosomal pathway is dependent on interactions of its C-terminus with the adaptor protein ARH[36]. The dependence on the megalin/cubulin complex for the constitutive reabsorption of albumin is evident in megalin knock-out mice[37] and cubulin deficient dogs[38], both of which have pronounced low molecular weight proteinuria and albuminuria.

In addition to the megalin/cubulin receptor complex, there is now increasing evidence derived from knockout models and disease states that the albumin endocytic complex consists of a number of accessory plasma membrane transport proteins[39]. There is a clear requirement for the v-H^+-ATPase, the pump that is responsible for the acidification of the endosome and lysosomes[39] (Figure 1). It has also been demonstrated *in vitro* using OK cells, that NHE3 plays a role in albumin uptake. This is based on several papers from the laboratory of Gekle and our own[29,40] showing that pharmacological inhibition of NHE3 with amiloride analogues or HOE694, or inhibition of NHE3 with cyclic adenosine monophosphate, results in pronounced decreases in albumin uptake[40]. Most convincingly, in NHE3 deficient OK cells, albumin uptake is effectively abolished while reintroduction of NHE3 normalises albumin uptake[40]. The most likely explanation for this effect of NHE3 is that it plays a role in the initial acidification of the nascent endosome, by acting to dissipate the high intraendosomal Na^+ concentration in exchange for cytosolic H^+. Interestingly, it has been reported that NHE3

binds megalin via a C-terminal tail interaction, suggesting that NHE3 may play an additional role as a molecular scaffold[41]. Although there are no reports of proteinuria in NHE3 knockout mice, this model is characterised by severe volume depletion, a significant reduction in glomerular filtration and an associated reduction in filtered protein[18]. Hence the specific role of NHE3 in Na^+ reabsorption in this model is difficult to ascertain.

The critical role of epithelial ion transport in tubular albumin transport is exemplified in Dent's disease, where inactivating mutations of the Cl^- channel, ClC-5, significantly inhibit tubular albumin reabsorption[42]. In patients with Dent's disease there are genetic abnormalities in ClC-5 leading to defects in channel trafficking or channel function[42,43] that in turn result in low molecular weight proteinuria as well as albuminuria due to defective proximal tubular protein reabsorption. A similar effect on tubular protein uptake is observed in ClC-5 knockout mice[44,45]. It has been considered that the main role of ClC-5 was to provide an anion shunt for the positive charge translocated by the v-H^+-ATPase into the endosome during acidification[46] (Figure 1). In support of this, in the kidneys of ClC-5 knockout mice, the uptake of markers of receptor-mediated and fluid phase endocytosis is severely impaired[44,45] and the acidification of the endosomes is decreased[44]. This finding is also consistent with the fact that many channels of the ClC family are believed to be involved principally in regulating intracellular Cl^- movement.

More detailed analysis, however, of the ClC-5 knockout mouse suggests that ClC-5, as well as acting as an anion shunt, plays an additional role in albumin endocytosis[45]. If ClC-5 were acting solely as an anion shunt, it would be predicted that the nascent endosome would be able to form and that the trafficking of the endosome would only be affected at a later (early endosome) stage when significant electrogenic H^+ movement occurs. This is particularly relevant when considering the role of electroneutral NHE3 exchange in initiating endosomal acidification, because this would remove the need for electrogenic transport of H^+ immediately following the budding of the endosome from the membrane. In support of this, there are reports indicating that the v-H^+-ATPase is not required for acidification of the early endosome[47].

In the brush borders of ClC-5 knockout mice exposed to the endocytic marker horseradish peroxidase, the marker was found to be trapped in a sub-plasmalemmal pre-endocytotic compartment and failed to enter the endosomal pathway[45]. This is somewhat surprising, since if the v-H^+-ATPase and hence anion shunt are not required during nascent endosome formation, it would be expected that the label would enter the early endosomal compartment. This raises the important point that the endocytotic defect may also be occurring earlier, at the formation of the nascent endosome. Further investigations in patients with Dent's disease showing that the loss of part of the C-terminus of ClC 5 also results in mistrafficking of the v-H^+-ATPase[48] and in ClC-5 knockout mice there are significantly reduced levels of megalin/cubulin at the plasma membrane also attributed to defective trafficking[49]. These findings strongly suggest that ClC-5 has an additional role in targeting key proteins involved in albumin uptake to the plasma membrane. Consistent with the role of ClC-5 at the plasma membrane, we have used surface biotinylation to demonstrate that ClC-5 is present at the cell surface (unpublished observations; cf [50]).

We have recently investigated a potential mechanism by which the C-terminal tail of ClC-5 can regulate albumin uptake. We found using a yeast 2-hybrid screen and glutathione S-transferase (GST)-pulldowns that ClC-5 interacted with the ubiquitously expressed actin binding protein cofilin[50] that is involved in actin depolymerization[51]. We reasoned that the passage of the nascent endosome through the cortical actin web required remodelling of the actin microfilament network. By phosphorylating cofilin with LIM kinase and thereby inhibiting the remodelling of the actin web we were able to inhibit albumin uptake in OK and LLC-PK1 cells[50]. This study demonstrates a critical role for ClC-5 via its C-terminal domain in mediating remodelling of actin microfilaments essential for albumin endocytosis. Our current hypothesis is that, although ClC-5 is expressed at the plasma membrane, the ion channel activity is redundant and that the protein plays a key role in mediating macromolecular complex assembly. This occurs via C-terminal tail scaffolding interactions with proteins directly involved in albumin endocytosis (v-H^+-ATPase and megalin/cubulin) as well as accessory proteins such as cofilin to form a localised and specialised endocytic complex (Figure 1).

Taken together, these data suggest that albumin uptake by the proximal tubule requires the assembly of a macromolecular complex at the plasma membrane that involves megalin/cubulin, ClC-5, NHE3 and v-H^+-ATPase. Determining the molecular composition and scaffolds associated with this complex and the precise regulatory mechanisms represents a key research focus in renal cell physiology.

Albumin uptake in diabetes mellitus

Patients with diabetes mellitus show a clear reduction in the capacity of the proximal tubule to reabsorb albumin[52], and this may even precede glomerular damage, demonstrating the importance of preventing tubular dysfunction early in the course of diabetes mellitus. Further evidence comes from studies in rat models of diabetes mellitus. Absolute tubular reabsorption of albumin is decreased in STZ rats[52] and ultrastructural studies have shown a decrease in albumin uptake and a reduction in the levels of megalin in the kidneys of STZ rats[53]. It is important to note that the presence of increased tubular protein overload leads to the development of inflammation and fibrosis[54] via activation of the nuclear factor-κB (NF-κB) transcriptional pathway[22]. This in turn induces the production of a number of proinflammatory stimuli such as regulated upon activation, normal T cell expressed and

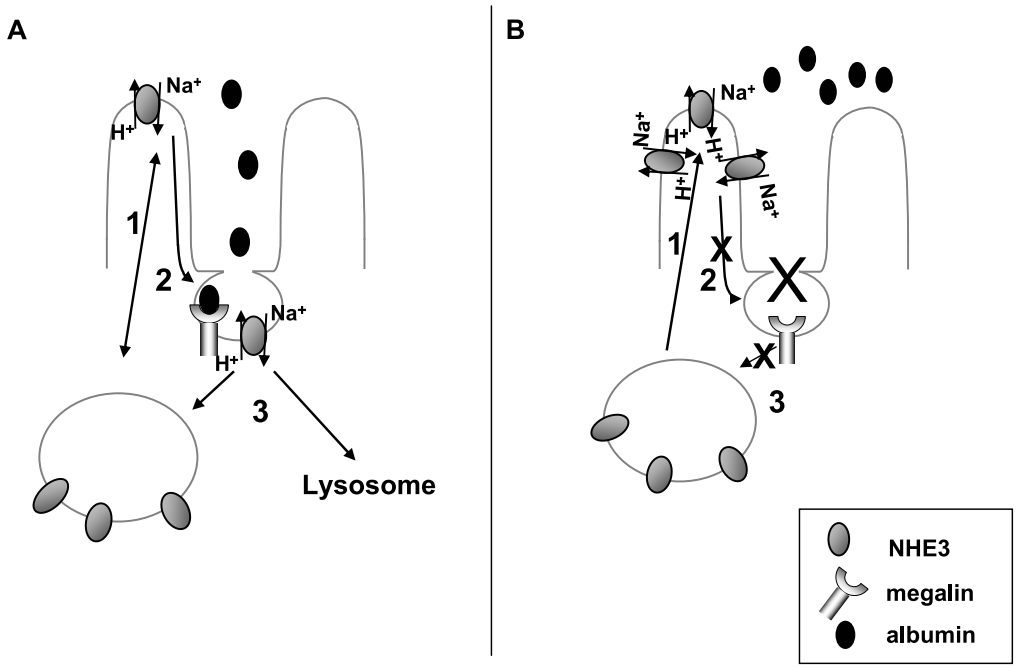

Figure 2. Possible alteration in NHE3 trafficking pathways in diabetes mellitus. Panel A: *Under normal conditions in proximal tubule cells the majority of NHE3 exists in recycling endosomes from where it is inserted into the microvilli (1) to reabsorb Na^+. Because of its role in albumin uptake, a proportion of the NHE3 may then translocate to the intravillar cleft where is associates with megalin/cubulin (2). This complex is then internalised and the NHE3 either returned to the recycling endosomes or degraded in the lysosomes (3).* Panel B: *The proteinuria associated with diabetes mellitus is in part due to an inhibition (x) of the normal albumin uptake pathway in the proximal tubule. As a result, the endocytosis of NHE3 via the megalin-associated pathway (2) is inhibited. However, insertion from the recycling endosomal pool is not affected (1), resulting in an accumulation of NHE3 in the microvillar pool and increased Na^+ reabsorption with proteinuria.*

secreted (RANTES), monocyte chemoattractant protein-1 (MCP-1) and transforming growth factor beta (TGF-β1[55]). TGF-β1 is regarded as the key inflammatory cytokine in diabetic nephropathy. Furthermore, it has been shown that elevated levels of intrarenal Ang II may in fact mediate the autocrine production of TGFβ1. In fact, a recent study in STZ rats has shown that Ang II blockade restored tubular albumin uptake, further highlighting the renin-angiotensin system in the development of diabetic nephropathy[56]. In the OK cell model of albumin uptake, it has been shown that TGF-β1 can regulate albumin uptake, by decreasing the binding, internalization and trafficking of the megalin/albumin complex[57]. Given that TGFβ1 levels are elevated in the diabetic kidney, this may provide a partial explanation for the molecular basis for the reduction in albumin uptake observed in vivo.

Interestingly, we have found that in OK cells, exposure to high glucose results in an increase in albumin uptake[29]. This effect is specific for glucose and not due to an osmotic effect and may occur as a result of the increase in NHE3 activity that is known to accompany exposure to high glucose[28,29]. It is likely that under these in vitro conditions, levels of autocrine TGFβ1 cannot reach the levels required to inhibit albumin uptake. Thus there appears to be a direct link between NHE3 activity and albumin uptake in OK cells. Furthermore, it has been shown in OK cells exposed to pathophysiological levels of albumin similar to those expected in diabetic nephropathy, that albumin uptake is reduced. This is due to a decrease in the number of albumin binding sites by an as yet undetermined mechanism that may involve altered rates of trafficking of megalin to or from the cell membrane[58]. It has been shown, however, in both primary cultures of human proximal cells[59] and OK cells[29,60], that exposure to high concentrations of albumin results in an increase in NHE3 expression and activity. A similar increase in NHE3 activity in response to increased tubular albumin has been reported in puromycin aminonucleoside nephrotic rats[61].

These data collectively suggest that NHE3 may exist in different functional pools, one associated with albumin uptake and the other involved in Na^+ reabsorption and not involved in albumin uptake (Figure 2). The evidence for the presence of NHE3 in two different pools in the proximal tubule brush border was presented in a recent review by McDonough and Biemesderfer[9]. One pool is located in the microvilli and the other in the intermicrovillar cleft where NHE3 co-localises with megalin. A number of studies have shown that NHE3 can shuttle between the two pools in

response to acute hypertension and other stimuli[9]. It is this association with megalin by an as yet uncharacterised molecular interaction that may explain the apparent role that NHE3 plays in albumin uptake. Furthermore, recent studies in OK cells have shown that a fraction of NHE3 is located in lipid rafts and that this may represent a different functional microdomain within the plasma membrane[62,63].

Based on the existence of different functional pools of NHE3, we postulate the following model that links increased Na^+ retention and proteinuria in diabetic nephropathy (Figure 2). (i) NHE3 exists primarily in subplasmalemmal pools where it is available for insertion in to the plasma membrane in response to numerous stimuli. (ii) A significant proportion of NHE3 is recycled/ removed from the membrane in conjunction with albumin, such that NHE3 opportunistically exploits the highly active albumin endocytic pathway for its recycling and that this represents a constitutive regulatory pathway for regulation of surface levels of NHE3. (iii) The NHE3 associated with megalin is not primarily involved in Na^+ reabsorption. (iv) When cells are exposed to high albumin, the endocytic pathway is reduced by an as yet uncharacterised mechanism, resulting in proteinuria (reduced albumin uptake) and a reduction in the internalisation rates of NHE3. (v) This in turn may result in a shift in the normal trafficking equilibrium of NHE3 with increased surface levels of NHE3 and potentiation of Na^+ retention. We are currently investigating the exact molecular mechanisms that may underlie the differences in NHE3 trafficking and albumin uptake in conditions of high glucose and high albumin.

It is also reported that exposure to high glucose results in significant alterations in the cytoskeleton in many different cell types. In terms of the kidney, studies in mesangial cells exposed to high glucose have shown pronounced rearrangements of the actin cytoskeleton that may contribute to the hyperfiltration associated with diabetes mellitus[64]. In addition, microarray analysis of mesangial cells have demonstrated altered levels of expression of actin regulatory proteins in response to high glucose[65]. It is also clear that both the trafficking of NHE3 and albumin uptake depend on an intact cytoskeleton[29,48,66]. In fact, we believe that it is critical to use the inhibition of albumin uptake by actin depolymerising agents to demonstrate that proximal tubule cells in culture are taking up albumin by a receptor-mediated pathway, as all cells have the ability to take up limited amounts of albumin by pinocytotic mechanisms. In proximal tubule cells, the actin at the microvillar core and in the terminal actin web must be in a constant state of remodelling to facilitate albumin endocytosis. Thus interactions between the membrane proteins and the cytoskeleton are essential for the regulation of ion transport activity and transporter/channel trafficking, control of vesicle movement and uptake as well as assembly of signalling and macromolecular complexes at the apical membrane[67,68].

It is important to note that, cultured proximal tubule cells do not have the extensive microvillar complex and intermicrovillar clefts characteristic of their in vivo counterparts (for review see[9]) despite retaining the core functional features of the proximal tubule, namely NHE3 dependent Na^+ uptake and megalin/cubulin mediated albumin uptake. Therefore, although much can be learned from studies in OK cells about endocytic complex assembly and regulation of albumin and NHE3, care must be exercised when extrapolating these data to the situation in the intact proximal tubule. Nevertheless, experiments in the cultured cell system can yield much valuable information regarding precise molecular interactions under defined conditions. For example, studies the on the role of NHE3 uptake in OK cells have highlighted an apparently facilitative function of NHE3 in albumin uptake that may not have been as readily identified in studies in the intact proximal tubule or in NHE3 knockout mice.

Conclusion

There is now compelling evidence for increased proximal tubule NHE3 activity contributing to the Na^+ retention that may underlie certain forms of hypertension including the hypertension often associated with diabetes mellitus. The existence of functionally different membrane domains and signalling/transporting complexes in the proximal tubule brush border may in part explain the relationship between increased Na^+ retention and reduced albumin uptake observed in diabetic kidney disease. It is becoming apparent that the location of NHE3 in different membrane domains is a critical determinant of NHE3 function. In addition, albumin uptake by the proximal tubule involves a macromolecular complex at the plasma membrane that involves megalin/cubulin, ClC-5, NHE3 and v-H^+-ATPase. Determining the molecular composition and scaffolds associated with this complex and the precise regulatory mechanisms represents a key research focus in renal cell physiology. A precise understanding of how these molecular interactions are altered in disease states such as diabetes mellitus will allow novel approaches to the diagnosis and management of diabetic kidney disease.

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Received 30 October 2003; in revised form January 21 2004. Accepted 29 January 2004.

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Differential neural control of glomerular ultrafiltration

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Summary

1. The renal nerves constrict the renal vasculature causing decreases in renal blood flow (RBF) and glomerular filtration rate (GFR). Whether renal haemodynamics are influenced by changes in renal nerve activity within the physiological range is a matter of debate.

2. We have identified two morphologically distinct populations of nerves within the kidney, which are differentially distributed to the renal afferent and efferent arterioles. TYPE I nerves almost exclusively innervate the afferent arteriole whereas TYPE II nerves are distributed equally on the afferent and efferent arterioles. We have also demonstrated that TYPE II nerves are immuno-reactive for neuropeptide Y while TYPE I nerves are not.

3. This led us to hypothesise that in the kidney, distinct populations of nerves innervate specific effector tissues and that these nerves may be selectively activated, setting the basis for the differential neural control of GFR. In physiological studies, we demonstrated that differential changes in glomerular capillary pressure occurred in response to graded reflex activation of the renal nerves, compatible with our hypothesis.

4. Thus, sympathetic outflow may be capable of selectively increasing or decreasing glomerular capillary pressure and hence GFR by differentially activating separate populations of renal nerves. This has important implications for our understanding of the neural control of body fluid balance in health and disease.

Introduction

Historical perspective

Opinion as to the importance of the renal nerves in controlling RBF and GFR has risen and fallen over the last 150 years. In the first study to demonstrate a role for the renal nerves in the control of renal function, Claude Bernard in 1859[1], transected the renal nerves and noted a marked diuresis, which he attributed to an increase in RBF. This and similar studies in the years following, demonstrating the phenomenon of denervation diuresis, dominated the understanding of the neural control of renal function (see [2]). During this period the renal nerves were thought to exert a profound effect on the regulation of RBF and GFR.

Yet 80 years later, opinion had swung full circle, when Homer Smith dismissed the renal nerves in his landmark book *The Physiology of the Kidney* [2] as having little importance in the control of renal function except in cases of severe stress. Smith damningly wrote of Bernard's study, stating that *"His conclusion is admittedly correct, but his experiment was unfortunate in two respects."*[2]. In the first instance, the development of clearance techniques to measure RBF and GFR demonstrated that changes in urine flow rate do not reflect changes in RBF. Secondly the anaesthesia and surgical stress to which the animals were subjected resulted in elevated basal levels of renal nerve activity. Bernard's finding of increased urine flow, probably was associated with an increase in RBF, but was due to the release of the kidney from the stress-induced increase in renal nerve activity. Smith's own studies, made painlessly in conscious and unstressed animals, failed to demonstrate any change in RBF or GFR following renal denervation. It was concluded that kidney function was not dependent on tonic renal sympathetic activity[2]. Soon after the first kidney transplantations were performed, the apparent lack of long-term effects on body fluid balance was taken as confirmation of the independence from nervous system control of renal vascular and tubular function[3].

However, in the 1970's there was a resurgence of interest in the neural control of renal function, sparked by quantitative analysis of the distribution and density of neuroeffector junctions in the kidney[4] and appreciation that transplanted kidneys rapidly re-innervate[5].

Significant role for nerves in renal function

Today it is widely accepted that changes in renal sympathetic nerve activity (RSNA) play a significant role in controlling body fluid homeostasis during normal daily activity and in the pathophysiology of many clinical conditions[6-8]. Whether this is primarily due to changes in renin release and tubular reabsorption, or also involves changes in RBF and GFR, is debated (see [9,10]).

In this review evidence is considered which supports the hypothesis that different populations of renal nerves selectively affect the afferent and efferent arterioles thereby allowing differential control of glomerular capillary pressure and hence single nephron glomerular filtration rate (SNGFR).

Control of glomerular ultrafiltration

A brief outline of the physiological basis of the control of glomerular ultrafiltration is necessary to understand how the renal nerves might contribute to its control. More detailed accounts can be found elsewhere (see [11]).

The primary force driving SNGFR is glomerular capillary pressure. Precise control of this pressure is important as significant falls in glomerular capillary pressure can lead to acute renal failure, whereas increased glomerular capillary pressure causes irreversible glomerular damage that leads to nephron loss and chronic renal disease[12].

The unique arrangement in the kidney of two resistance vessels in series, the afferent and efferent arterioles, allows fine regulation of pressure in the glomerular capillaries[13,14]. RBF only becomes an important factor in determining SNGFR under conditions of filtration equilibrium, which is not the normal physiological state[13,14]. Thus, glomerular capillary pressure and therefore SNGFR will increase if the pre (afferent) to post (efferent) glomerular resistance ratio decreases and decrease if this resistance ratio increases.

Importantly, for the majority of glomeruli the resting diameter of the efferent arteriole is smaller than the afferent arteriole[15-18]. Since resistance is inversely proportional to the fourth power of the radius this explains, in part, how this relatively small, sparsely muscled vessel can counterbalance the effects of constriction of the bigger, more muscular afferent arteriole (see [17] for a more detailed explanation). For juxtamedullary glomeruli - those 10% of glomeruli, whose efferent arterioles descend into the medulla to form the vasa recta - the situation is different since these efferent arterioles are as large if not larger than their afferent counterparts. Therefore the control of glomerular capillary pressure may well be different in juxtamedullary nephrons.

SNGFR can also be influenced by alterations in the glomerular capillary ultrafiltration coefficient (K_f), which represents the product of the glomerular capillary surface area available for filtration and hydraulic conductivity. K_f has been shown to decrease in response to a number of vasoactive stimuli, though the mechanisms are not well understood (see [11]).

Neural control of renal function

Renal innervation

The kidney receives an extensive sympathetic innervation. While it is generally agreed that all the major structural elements of the kidney are innervated, including vascular smooth muscle cells, renin secreting cells, mesangium and tubules (proximal, distal and loop of Henle)[19,20], the relative density of the innervation of each tissue type has been disputed[20]. Whether functionally specific or non-specific renal sympathetic nerve fibres innervate the effector cells has also been questioned. Barajas concluded that the sympathetic innervation of the kidney was diffuse and non-specific, based on observations that each sympathetic axon made contact with multiple effector tissues (see [19]). These studies powerfully influenced how the nerves were thought to control renal function (see [21]). However, the definition of a neuroeffector contact in Barajas's studies is now considered very broad. Varicosities that were separated from the effector cell by up to 300 nm and in which two layers of basal lamina were present were included (see [19]).

Our definition of a neuroeffector junction is much more specific[20,22]. The varicosities along an axon can be divided into contacting and non-contacting. Contacting varicosities form specialized junctions with the effector cell; neuroeffector junctions. The vesicles are organized within the varicosity in clusters associated with the region on the membrane that is in contact with the effector cells. At the point of contact, the varicosity and effector cell are separated by a gap that is less than 100 nm[20,22]. Consequently, contacting varicosities release neurotransmitters directly onto junctional receptors rather than relying on diffusion of the transmitter to receptors across the surface of the smooth muscle cell membrane[20,22]. Based on this definition of a neuroeffector junction, we re-examined the innervation of the kidney and drew vastly different conclusions to those drawn from the studies of Barajas (see [19]).

Two structurally distinct types of sympathetic axons.

Using three dimensional reconstruction ultrastructural analysis of serial thin sections to examine the innervation of the juxtaglomerular region, we identified two ultrastructurally distinct types of sympathetic axons[23]. TYPE I axons were large in diameter with atypical varicosities and TYPE II axons resembled those innervating blood vessels in other organs, with typical fusiform varicosities[23]. These axon types were identified in rats and rabbits[23]. At the time the functional significance of two axon types was unknown, though conduction velocities would be expected to be different. Later, in support of our study, another study demonstrated that there was a bimodal distribution in the diameters of the renal nerves with different conduction velocities [24].

Innervation density of TYPE I & II axons

Next we described the distribution and density of neuroeffector junctions made by these two types of axons[20]. Several important findings were made: (i) The sympathetic axons were located in regions adjacent to the renal vasculature and therefore primarily the arterial vessels were innervated. However, the majority of tubular tissue in the cortex was not innervated. (ii) The afferent arteriole was the most densely innervated tissue. The afferent arterioles were 3 times more densely innervated than the efferent arterioles (Fig. 1 & 2). (iii) There was little evidence for individual axons innervating more than one effector cell type. (iv) Most significantly, it was shown that TYPE I axon varicosities made contact almost exclusively with afferent arterioles whereas TYPE II axons innervated both arterioles at similar densities (Fig. 1 & 2).

This finding was of great potential significance, and was also recognized as such by others, being rapidly incorporated into standard textbooks on the kidney[25,26]. It raised the possibility that TYPE I and II axons originated from different populations of neurons.

Chemical coding of distinct nerve populations

The presence of distinct combinations of immuno-histochemically detectable substances can be used to identify populations of nerves serving different functions[27,28]. On this basis, we have recently shown that neuropeptide Y is located in TYPE II axons whereas TYPE

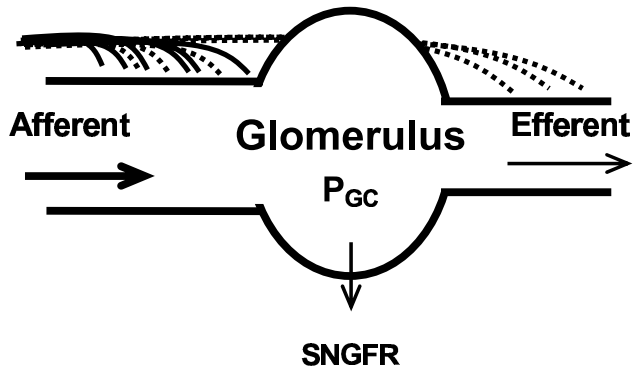

Figure 1. *Diagram representing the relative TYPE I (solid line) and TYPE II (dashed line) axon innervation density on afferent and efferent arterioles. The afferent arteriole is 3 times more densely innervated than the efferent arteriole. TYPE I axons (solid lines) almost exclusively innervate the afferent arteriole. TYPE II axons (dotted lines) are equally distributed on the afferent and efferent arterioles. (P_{GC}, glomerular capillary pressure. SNGFR, single nephron glomerular filtration rate).*

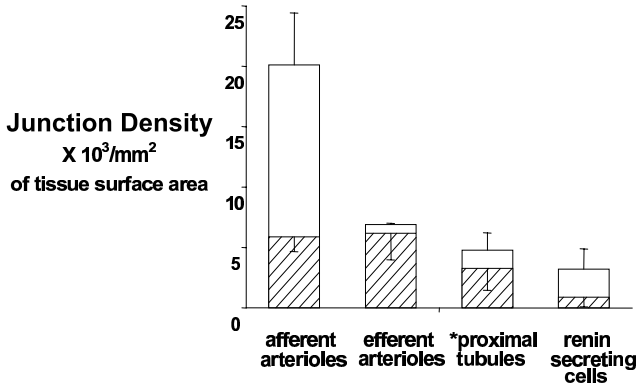

Figure 2. *Stacked bar graph of the density of neuroeffector junctions of TYPE I (open bar) and TYPE II (hatched bar) axons on the afferent arterioles, efferent arterioles, proximal tubules (*only those adjacent to the afferent arterioles were innervated) and renin secreting cells. The combined bar equals the total junction density on each effector tissue.*

I axons lack neuropeptide Y[29]. Our findings are in good accord with the study of Reinecke *et al.*[30] who reported that the density of neuropeptide Y positive terminals was very similar on the afferent and efferent arterioles; that is, similar to the distribution of TYPE II axons. This provides further evidence that TYPE I and II axons originate from separate populations of neurons. The search for a neuropepetide specific to TYPE I axons is on going.

Hypothesis

On the basis of our morphological and immunohistochemical evidence, we hypothesised that different patterns of sympathetic outflow to the kidney may evoke selective changes in pre- and post-glomerular vascular resistance to regulate GFR.

We hypothesise, based on the distribution of the TYPE I and TYPE II nerves on the afferent and efferent arterioles that (see Fig. 1 & 3), (i) Selective TYPE I axon activation would result in pre-glomerular vasoconstriction, reduced RBF, a reduction in glomerular capillary pressure and a fall in GFR. (ii) Selective TYPE II axon activation would result in pre- and post-glomerular vasoconstriction and decreased RBF. However, the effect on resistance would be greater on the efferent arteriole, since it is a smaller vessel (Poiseuille's law), leading to little effect on glomerular capillary pressure resulting in the maintenance of GFR[17]. (iii) Activation of both TYPE I and II axons would cause a predominant decrease in pre-glomerular vascular resistance due to the greater innervation density of the afferent arteriole. We have pursued this possibility in physiological studies outlined below.

Differential sympathetic outflow

The sympathetic nervous system is capable of producing selective changes in efferent outflow to different organs (see [31-33]). Increasing knowledge of central autonomic nervous system organisation, indicates that the output to different sympathetic pre-ganglionic neurons depends on the relative contributions of a wide range of brain nuclei and on the particular pattern of inputs to those nuclei (baroreceptor, chemoreceptor, somatic receptors and inputs from all areas of the brain)[31]. We are proposing within the kidney, as has been demonstrated in other organs (eg there are at least 3 distinct types of sympathetic neurons in the gut[28]), that there is further differentiation of the signal such that specific effector tissues may be selectively activated, depending on the nature and severity of the stimulus. In the literature there is limited and conflicting evidence as to whether subpopulations of renal post-ganglionic nerves can selectively regulate different renal functions[34,35].

Physiological studies

Current views on the neural regulation of renal function rely mainly on data from electrical stimulation studies, or even the effects of simple acute denervation. It has been widely accepted that individual renal nerves innervate multiple tissues (vascular smooth muscle, renin secreting cells and proximal tubules)[19] and that renal function is affected entirely by the frequency of their firing, with low to moderate frequencies stimulating increased renin release and sodium reabsorption and only high frequencies stimulating a decrease in renal blood flow and GFR (see [21]). According to this view renal sympathetic nerve activity (RSNA) is generally too low to influence renal vascular resistance and glomerular ultrafiltration under normal physiologic conditions[21]. This does not accord however with a large body of physiological and clinical evidence that suggests that renal hemodynamics are under the control of RSNA during daily events (see [9]).

Not surprisingly, since the afferent arteriole is much more densely innervated than the efferent arteriole[20], electrical stimulation of the renal nerves results in a predominant increase in pre-glomerular resistance[36]. This causes glomerular capillary pressure and GFR to decrease, as predicted when both TYPE I and II nerves are fired simultaneously (see Fig 3). These studies therefore shed no light on the possible effects of selective physiological recruitment of different populations of renal nerves on the renal resistance vessels.

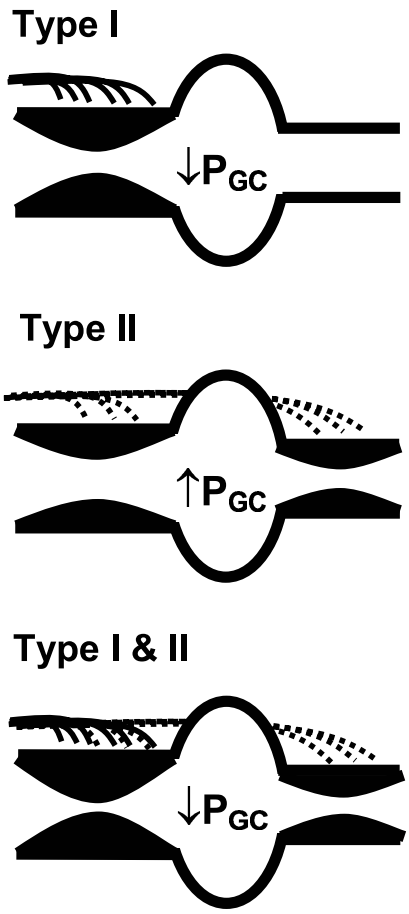

Figure 3. *Diagram demonstrating the hypothetical effects of selective activation of TYPE I (upper panel), TYPE II (middle panel) or both TYPE I and II (lower panel) axons innervating the afferent and efferent arteriole, on glomerular capillary pressure (P_{gc}). See text for explanation.*

RSNA varies in both the frequency (reflecting the rhythms of the central generating circuits and baroreflex input) and amplitude of its discharge (reflecting the relative number of activated nerves)[37,38]. Thus, whereas electrical stimulation activates all nerves simultaneously, it is now evident that relatively few individual nerves are active at rest and that the number of nerves activated during physiological bursts of nerve activity varies widely[39]. Physiological activation of the renal sympathetic nerves is therefore fundamentally different to electrical stimulation.

The influence of RSNA on GFR

A number of studies have examined the kidney's response to reflex activation of the renal sympathetic nerves. Again not surprisingly, in response to severe increases in RSNA, when many nerves are firing, RBF and GFR decrease (see [21]), indicative of TYPE I and/or II nerve firing (see Fig. 3). However, in several studies no change in GFR was reported in response to moderate increases in RSNA[40-43]. It is quite possible that in these studies subtle changes in pre- and post-glomerular vascular resistances were occurring to maintain glomerular capillary pressure and GFR. However, no study measured glomerular capillary pressure to verify such a conclusion.

Renal micropuncture allows discrimination of pre- & post-glomerular vascular resistance.

At this time, it is not possible to identify individual Type I versus Type II nerves *in vivo*, and thus selectively record or stimulate these neurons. However, we do have tools whereby we can determine whether the pattern of changes in pre-and post-glomerular vascular resistance in response to reflex stimulation of RSNA is presumptive of TYPE I nerve recruitment, TYPE II nerve recruitment or both. *In vivo* micropuncture is a challenging and time consuming procedure, but it is the only means whereby pressure can be directly measured in the glomerular capillaries and is thus essential in studies evaluating the contribution of the pre-and post-glomerular vessels to changes in renal hemodynamics and glomerular function [13,14].

Differential recruitment of TYPE I and II nerves

To begin to investigate this hypothesis, we examined the effects of physiologically induced increases in renal sympathetic nerve activity (RSNA) in response to graded hypoxia on pre- and post-glomerular vascular resistances in anaesthetised rabbits[10]. We chose hypoxia to reflexly increase RSNA because we had previously shown that it produces graded increases in the amplitude of renal nerve firing (i.e. graded recruitment of individual nerves)[44]. Hypoxia has the further advantage that it does not significantly alter arterial pressure in the rabbit, thereby avoiding confounding autoregulatory effects on renal haemodynamics[44]. In the first study of its kind we measured simultaneously glomerular capillary pressure, renal nerve activity and whole kidney function, while subjecting the rabbits to different degrees of hypoxia[10]. The results were clear-cut, and compatible with the hypothesis that TYPE I and II axons can be differentially activated (see Fig. 3).

We found that moderate (14% O_2) and severe (10% O_2) hypoxia increased total RSNA by 60 % and 170 % respectively, chiefly by increasing the amplitude of the sympathetic bursts rather than their frequency. Moderate hypoxia decreased RBF (26%), increased glomerular capillary pressure and maintained GFR (Fig. 4). Both pre- and post-glomerular vascular resistances were increased;

but there was a predominant effect on the post-glomerular vasculature. This greater effect on the efferent arteriole, when the TYPE II innervation density is similar on both afferent and efferent arterioles, can be explained on the basis of Poiseuille's Law and the smaller resting diameter of the efferent arteriole[10]. In short, the recruitment of nerves in response to moderate hypoxia appeared to be predominantly TYPE II nerves (Fig. 3). In contrast, severe hypoxia decreased RBF (56%), with a significant fall in glomerular capillary pressure and GFR (Fig. 4). This pattern reflects a substantially greater pre-glomerular than post-glomerular vasoconstriction that is compatible with the further recruitment of nerves by severe hypoxia being predominantly TYPE I nerves (Fig. 3). These results provide evidence that different levels of reflexly induced increases in RSNA may differentially control pre- and post-glomerular vascular resistance, compatible with selective activation of TYPE I and II renal sympathetic nerves.

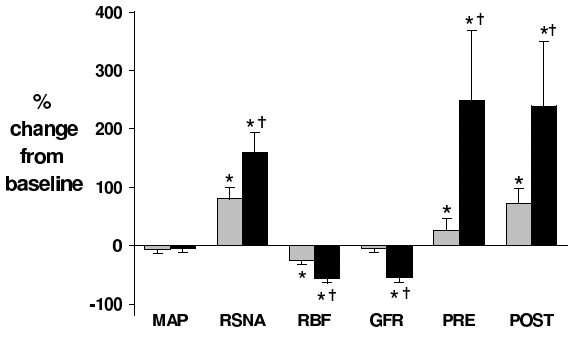

Figure 4. *Responses to moderate (14% O_2; grey) and severe (10% O_2, black) hypoxia in anaesthetised rabbits. Values (means ± s.e.m. n = 7) are the percentage change from baseline (room air, 21% O_2) for mean arterial pressure (MAP), renal sympathetic nerve activity (RSNA), renal blood flow (RBF), glomerular filtration rate (GFR) and pre (PRE) and post (POST glomerular vascular resistance.* * P < *0.05 change from baseline,* † P < *0.05 14% O_2 vs 10% O_2.*

We are confident that the effects of hypoxia were mediated via the renal nerves as we have previously demonstrated the absence of any renal action of hypoxia following renal denervation[42,45]. However, neurally mediated renin release may have contributed to the response to increased RSNA, as renin cells are innervated by both TYPE I and II axons[20]. In particular, the renal response during moderate hypoxia might be explained on the basis of an increase in renin release being responsible for the rise in post-glomerular resistance. Though plasma renin activity was not increased in response to moderate hypoxia, intrarenal effects cannot be discounted[10].

Contribution of ANGII

The question of the involvement of the renin-angiotensin system in the response to moderate (14% O_2) hypoxia was investigated. The renin-angiotensin system was rendered unresponsive by the simultaneous infusion of an angiotensin converting enzyme inhibitor and ANGII to restore normal blood pressure ('ANGII clamp'). Measurements were made in rabbits receiving either the 'ANGII clamp' or vehicle infusion before (room air, i.e. 21% O_2) and during moderate hypoxia (14% O_2) [46]. As seen in our previous study[10], in the vehicle group RSNA increased in response to 14% O_2, and this decreased RBF, without effecting GFR or arterial pressure. Though the response was attenuated in the 'ANGII clamp' group, glomerular capillary pressure increased in both the vehicle and 'ANGII clamp' groups during 14% O_2 (Fig. 5). These results are consistent with the notion that direct actions of TYPE II nerves on the efferent arterioles are responsible in part for the increase in post-glomerular resistance in response to 14% O_2 [46]. These results further support our hypothesis that different populations of renal nerves selectively control pre- and post-glomerular resistance and hence glomerular pressure and ultrafiltration. Future studies will extend these findings by examining the renal microvascular response to stimulation of central nuclei involved in cardiovascular control[31].

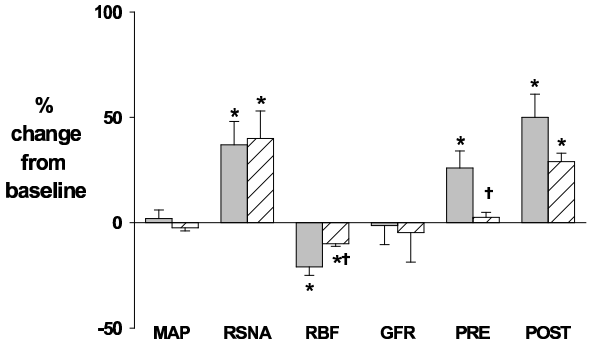

Figure 5. *Responses to moderate (14% O_2) hypoxia in anaesthetised rabbits treated throughout the study with vehicle (grey) or 'ANGII clamp' (infusion of an angiotensin converting enzyme inhibitor plus ANG II to restore blood pressure to normal; hatched). Values (means ± s.e.m. n = 6) are the percentage change from baseline (room air, 21% O_2) for mean arterial pressure (MAP), renal sympathetic nerve activity (RSNA), renal blood flow (RBF), glomerular filtration rate (GFR) and pre (PRE) and post (POST glomerular vascular resistance.* * P < *0.05 change from baseline,* † P < *0.05 vehicle vs ANGII clamp.*

Perspective

Alterations in renal sympathetic nerve activity produce important effects on renal function, which contribute to the kidney's main task of regulating body fluid

balance. Our data suggest that there are functionally specific post-ganglionic renal nerves that can be selectively activated. Based on our evidence, we propose that TYPE II nerves predominate in the physiological control of arteriole resistance to maintain GFR constant during daily activity, whereas Type I nerves play a role when the animal is under stress (hemorrhage, dehydration or exercise), when blood flow is required for other organs at the expense of renal function. Overactivity of the renal nerves has been implicated in the pathophysiology of hypertension[7], congestive heart failure[6] and chronic renal failure[8]. Studies examining whether one or other of the populations of renal nerves are involved in these diseases offer possibilities of new therapeutic targets.

Acknowledgements

This work was supported by grants from the National Health and Medical Research Council of Australia (97/0426, 236821). Dr Kate M Denton is the recipient of a Research Fellowship awarded by the Foundation for High Blood Pressure Research, Australia. The technical assistance of Rebecca Flower is gratefully acknowledged.

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Received 30 October 2003; in revised form January 14 2004. Accepted 29 January 2004.

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Proceedings of the Australian Physiological and Pharmacological Society (2004) ***34:*** *93-104* *http://www.apps.org.au/Proceedings/34/93-104*

Neural control of renal medullary perfusion

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Summary

1. There is strong evidence that the renal medullary circulation plays a key role in long-term blood pressure control. This, and evidence implicating sympathetic overactivity in development of hypertension, provides the need for understanding how sympathetic nerves affect medullary blood flow (MBF).

2. The precise vascular elements that regulate MBF under physiological conditions are unknown, but likely include the outer medullary portions of descending vasa recta, and afferent and efferent arterioles of juxtamedullary glomeruli, all of which receive dense sympathetic innervation.

3. Many early studies of the impact of sympathetic drive on MBF were flawed, both because of the methods used for measuring MBF, and because single and often intense neural stimuli were tested.

4. Recent studies have established that MBF is less sensitive than cortical blood flow (CBF) to electrical renal nerve stimulation, particularly at low stimulus intensities. Indeed, MBF appears to be refractory to increases in endogenous renal sympathetic nerve activity within the physiological range in all but the most extreme cases.

5. Multiple mechanisms appear to operate in concert to blunt the impact of sympathetic drive on MBF, including counter-regulatory roles of nitric oxide, and perhaps even paradoxical angiotensin II-induced vasodilatation. Regional differences in the geometry of glomerular arterioles are also likely to predispose MBF to be less sensitive than CBF to any given vasoconstrictor stimulus.

6. Failure of these mechanisms would promote reductions in MBF in response to physiological activation of the renal nerves, which could in turn lead to salt and water retention and hypertension.

Introduction

'Neural control of the capillary circulation in specific regions of the kidney has not been adequately studied'. This statement from Pomeranz *et al.* in 1968[1] could reasonably have been made almost 30 years later, with little progress being made in this field in the intervening period. However, renewed activity in this area since 1995[2] has increased our understanding of the influence of renal sympathetic drive on regional kidney blood flow. As we will describe in this review, there is now strong evidence that medullary blood flow (MBF) is less sensitive than cortical blood flow (CBF) to increases in renal sympathetic drive within the physiological range. This has important implications for the control of renal function, and in particular, the long-term regulation of arterial pressure. Nitric oxide appears to play a critical role in protecting the renal medulla from ischaemia due to renal nerve activation, but is not the only factor involved. For example, unique structural aspects of the medullary circulation probably contribute, and angiotensin II may have a surprising role as a counter-regulatory vasodilator within the medullary microcirculation. There is also the potential for neurochemical differences between nerves innervating vascular elements controlling MBF and CBF to contribute.

The aim of this review is to examine the mechanisms, and implications, of the neural regulation of MBF. However, we must first discuss three important issues: the unique vascular architecture of the kidney that underlies the differential control of MBF and CBF, the physiological imperatives of precise regulation of MBF, and the nature of the renal sympathetic innervation and its role in blood pressure control. We will then consider the evidence of differential neural control of CBF and MBF, and the potential mechanisms that underlie it.

The renal medullary circulation: structure and function

The blood supply to the renal medulla arises from the efferent arterioles of juxtamedullary glomeruli, which comprise ~10% of all glomeruli in the kidney (Figure 1). Thus, while all blood flow to the kidney enters the renal cortex, only ~10% of this enters the renal medulla. In rats and dogs, reliable estimates of regional kidney blood flow have ranged from 2.6-7.4 ml/min/g in the cortex, 1.3-3.2 ml/min/g in the outer medulla, and 0.2-5.9 ml/min/g in the inner medulla[3]. Although these estimates show considerable variability between various studies (and species), it is widely regarded that blood flow per unit tissue weight in the outer and inner medulla is approximately 40% and 10%, respectively, that in the cortex. The maintenance of a relatively low MBF appears to be critical for maintaining the cortico-medullary solute gradient, and so urinary concentrating mechanisms[3]. On the other hand, because the renal medulla is a hypoxic environment even under normal conditions, there must be some trade off in the control of MBF, between maintenance of the cortico-medullary solute gradient (and so normal tubular function), and the supply of oxygen within the renal medulla (Figure 2). As will be described in detail below (see *MBF and blood pressure control*), there is also strong evidence that the level of MBF is a key factor in long-term control of blood pressure.

The precise vascular elements that regulate MBF under physiological conditions remain unknown. However, from a theoretical perspective changes in vascular resistance in juxtamedullary arterioles, or in downstream vascular elements within the medulla itself (eg, outer medullary descending vasa recta), could lead to large

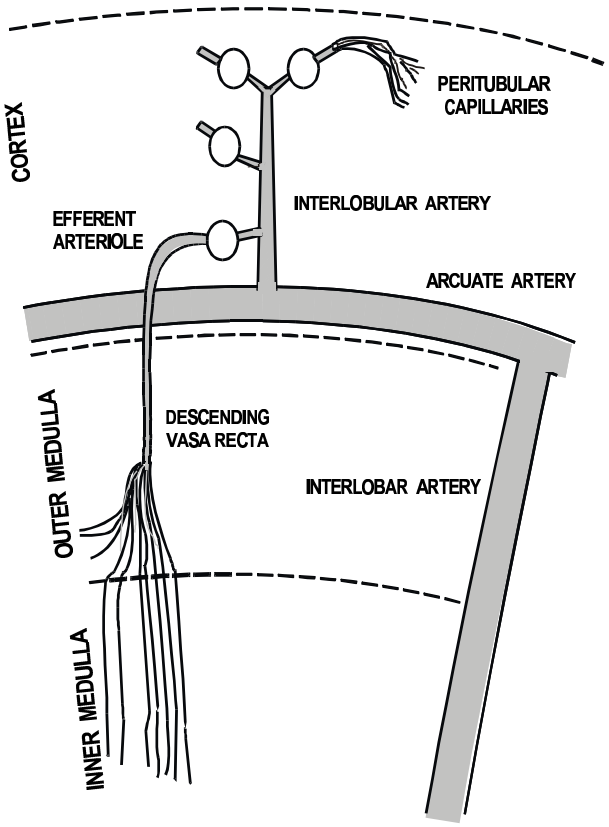

Figure 1. *Schematic diagram of the architecture of the renal vasculature, and the extent of renal innervation to various vascular elements (shaded). Based on original figures by others[3,71]. Innervation data adapted from Barajas & Powers[24].*

changes in MBF without significant alterations in total CBF. On the other hand, because juxtamedullary afferent arterioles arise near the origin of interlobular arteries (Figure 1), changes in interlobular artery calibre would be expected to impact less on MBF (and juxtamedullary cortical blood flow) than on the bulk of CBF.

Heterogeneity of the geometry of glomerular arterioles may also contribute to the differential regulation of CBF and MBF. Afferent and (particularly) efferent arterioles of juxtamedullary glomeruli have considerably greater calibre than their counterparts in the mid- and outer-cortex[4] (Figure 1). Because vascular resistance is inversely proportional to vessel radius to the power of 4, comparable changes in vessel radius result in lesser absolute change in vascular resistance in the larger juxtamedullary arterioles, than in their counterparts in other regions of the cortex[4].

MBF and blood pressure control

Although the precise aetiology of essential hypertension remains unknown, there is persuasive evidence that the initial trigger resides within the kidney[5]. One line of evidence in support of this notion arose from the seminal work of Guyton and colleagues[6], showing that the pressure diuresis/natriuresis mechanism provides a 'non-adapting' feedback system by which arterial pressure can be controlled in the long-term. The relationship, between renal perfusion pressure and salt and water excretion (pressure diuresis/natriuresis), is set at higher pressures in all forms of hypertension that have been studied, and hypertension can be ameliorated by treatments that restore this relationship towards normal. Another important line of evidence comes from studies of renal transplantation between hypertensive and normotensive subjects. Both in rats and humans, there is good evidence that 'the blood pressure follows the kidney'[7]. That is, when a kidney from a normotensive subject is transplanted into a hypertensive subject, arterial pressure falls. Conversely, when the kidney from a hypertensive subject, or a normotensive subject genetically pre-disposed to hypertension, is transplanted into a normotensive subject, hypertension develops.

In a series of elegant studies reviewed in detail previously[8-11], Cowley, Roman, Mattson and colleagues have provided persuasive evidence that MBF is a critical factor in the long-term control of arterial pressure. They have utilised a conscious rat model in which CBF and MBF are measured chronically using implanted optical fibres, while vasoactive agents are administered directly into the renal medulla. Chronic medullary interstitial infusion of vasoconstrictors, at doses that reduce MBF, produce hypertension, whereas similar infusions of vasodilators that increase MBF can ameliorate hypertension. This effect seems to be mediated through alterations in the pressure diuresis/natriuresis relationship, which is shifted to higher pressures by both chronic and acute medullary interstitial infusions of vasoconstrictors, and shifted to lower pressures by medullary interstitial infusions of vasodilators. We have confirmed some of these observations in a different species, showing that acute medullary intersititial (but not intravenous) infusion of noradrenaline shifts the pressure diuresis/natriuresis relationship to higher pressures in anaesthetized rabbits[12,13].

The precise mechanisms by which reductions in MBF shift the pressure diuresis/natriuresis relationship to higher pressure remain a matter of controversy. Cowley and colleagues have developed the hypothesis, for which there is considerable experimental support,[8-11] that increases in MBF in response to increased renal perfusion pressure actually mediate pressure diuresis/natriuresis. Increased vasa recta capillary hydrostatic pressure (secondary to increased vasa recta blood flow) will result in increased medullary interstitial hydrostatic pressure, which will be transmitted throughout the kidney because of the low compliance of the kidney due to the presence of the renal capsule. Increased renal interstitial hydrostatic pressure reduces sodium reabsorption in a number of segments of the nephron, probably in part through enhanced back-leak along paracellular pathways. However, the integrity of this hypothesis depends on the idea that MBF, unlike total renal blood flow (RBF) and CBF, is relatively poorly autoregulated. The degree to which MBF is autoregulated remains a matter of controversy,[14,15] probably in part

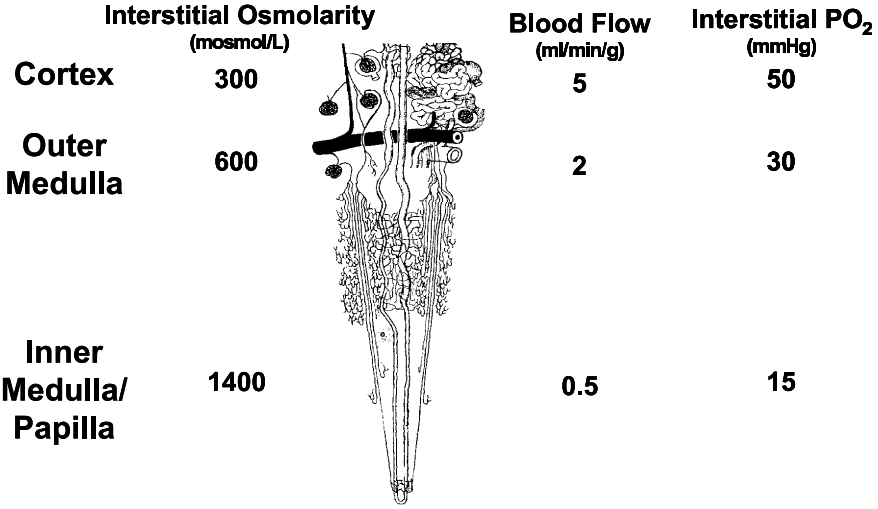

Figure 2. *The trade-off between maintenance of the cortico-medullary solute gradient and medullary hypoxic damage. Diagram of the renal vasculature adapted from Beeuwkes & Bonventre[73]. Data relating to interstitial osmolarity, blood flow and interstitial PO_2 compiled from Vander[74], Pallone* et al.*[3], and Lübbers & Baumgärtl[75] respectively.*

because of limitations in available methods for estimating MBF.

Renal nerves and blood pressure control

There is clear evidence that renal sympathetic drive is increased during the development of hypertension both in the spontaneously hypertensive rat (SHR) and in human essential hypertension. Thus, basal post-ganglionic sympathetic nerve activity[16], and emotional stress-induced increases in post-ganglionic sympathetic nerve activity and reductions in sodium excretion[17], are enhanced in SHR compared with normotensive Wistar Kyoto control rats (WKY). Furthermore, renal sympathetic drive, as measured by noradrenaline spillover, is also increased in human essential hypertension[18]. Increased renal sympathetic drive appears to contribute to the pathogenesis of hypertension, since in SHR chronic bilateral renal denervation, achieved by repeated denervation between weeks 4 and 16 after birth, blocks 30-40% of the expected progressive elevation of arterial pressure[19]. A similar regimen of bilateral renal denervation in WKY has no effect on arterial pressure[19].

The precise mechanisms by which increased renal sympathetic drive contributes to the pathogenesis of hypertension remain unknown. The fact that reductions in MBF can shift the pressure diuresis/natriuresis relationship to higher pressures (right-ward shift), which if maintained chronically produces hypertension, provides the impetus for our interest in the neural control of MBF.

Innervation of vascular elements controlling MBF

The origin of the efferent sympathetic innervation of the kidney differs among species, but in general arises from multiple ganglia of the celiac plexus, the lumbar splanchnic nerve and the intermesenteric plexus[20]. Post-ganglionic nerves enter the kidney in association with the renal vasculature, and follow the course of the renal arterial tree as it branches to form interlobar, arcuate, and interlobular arteries. These neurones in turn innervate the afferent and efferent arterioles, and the outer medullary portions of descending vasa recta, but not vascular elements within the inner medulla and papilla[21] (Figure 1). Consistent with these anatomical observations, juxtamedullary afferent and efferent arterioles of the rat hydronephrotic kidney constrict in response to renal nerve stimulation[22].

Previous studies of regional differences in innervation density within the kidney indicate that juxtamedullary afferent and efferent arterioles, and their associated outer medullary descending vasa recta, are densely innervated. For example, McKenna & Angelakos found the juxtamedullary cortex and outer medulla to have the greatest concentration of noradrenaline within the dog kidney, levels being ~40-60% less in the mid- and subcapsular-cortex, and low in the inner medulla[23]. Barajas and Powers provided more direct evidence of dense juxtamedullary vascular innervation, using autoradiography to detect uptake of exogenous [^{3}H]-noradrenaline (presumptively by sympathetic nerves) in rat kidney[24]. They found greater density of autoradiographic grains on afferent, compared with efferent arterioles throughout the cortex, but autoradiographic grain density was similar in each of these vascular elements in the outer- mid- and juxtamedullary cortex. Quantitative analysis of the innervation density of outer medullary descending vasa recta was not included in their study, although the amount of autoradiographic grains overlapping the vasculature was greater in the outer stripe of the outer medulla than in any other kidney region. It must be born in mind, however, that the techniques that have been applied to this problem have considerable limitations. Most evidence suggests that

sympathetic neurotransmission in blood vessels occurs chiefly via specialised neuromuscular junctions, at which varicosities form a close contact (< 100 nm) with arteriolar smooth muscle cells[25]. In the rabbit kidney, ~80% of sympathetic varicosities within the arteriolar region form these specialised neuromuscular junctions[26]. On the other hand, it has also been argued that sympathetic neurotransmitters can also act at some distance from their site of release within the kidney, particularly in the control of tubular function[20]. Nevertheless, the relative distribution of specialised neuromuscular junctions in vascular elements controlling CBF and MBF would better reflect the density of 'functional' sympathetic innervation in the renal vasculature, than measures of tissue noradrenaline content *per se*[23], or the density of sites of noradrenaline uptake[24]. There is a need, therefore, for further detailed studies of the innervation of vascular elements controlling MBF and CBF.

Neurochemistry of renal sympathetic nerves

Most evidence suggests that the predominant neurotransmitter in renal sympathetic nerves is noradrenaline. Thus, while dopamine also appears to be present in these nerves as a precursor of noradrenaline synthesis, there is little compelling evidence of specific dopaminergic nerves within the kidney[20]. Moreover, while acetylcholine is found within the kidney, it appears not to be associated with renal nerves[20]. Nevertheless, there is now strong evidence that co-transmitters, including neuropeptide Y and ATP participate in renal sympathetic neurotransmission[20] and partially mediate renal nerve stimulation induced-reductions in global RBF[27-30]. Other neurotransmitters, including vasoactive intestinal polypeptide and neurotensin, have been identified within the renal vasculature[31], and galanin has been identified in a proportion of the neurons innervating the kidney[32]. Their roles in renal sympathetic neurotransmission and in regulating renal function remain to be determined. Neuropeptide Y[31] and its binding sites[33], and also neurotensin and vasoactive intestinal polypeptide[31] have been localised to vascular elements of the medullary circulation (including juxtamedullary afferent and efferent arterioles), raising the possibility that these sympathetic co-transmitters could contribute to the neural control of MBF.

Neural control of MBF: early studies

All available methods for estimating regional kidney blood flow have limitations that must be considered in the interpretation of experimental data[3,34]. Methods used in early studies of the control of MBF, based on para-aminohippuric acid clearance, washout of diffusible tracers such as ^{85}Kr, H_2, and heat (thermodilution), renal extraction of diffusible indicators such as ^{42}K and ^{86}Rb, indicator transit time, albumin accumulation and microspheres have been shown to be (more or less) invalid from either practical or theoretical standpoints[3,33]. For the most part, these methods are also limited by the fact that they do not provide 'real time' measurements of blood flow in individual animals. A further limitation of many early studies of the neural control of intrarenal blood flow is that they often employed single, intense stimuli, well beyond what one might consider to be physiologically relevant. However, it is worthwhile for us to briefly consider the results of studies using these techniques, because they allow us to appreciate both the heroic efforts of earlier investigators, and the evolution of our understanding, of the neural control of intrarenal blood flow.

Trueta *et al.* were the first to study this issue (in 1947), using the intrarenal distribution of injected radiocontrast material and Indian ink as markers of blood flow in anaesthetized rabbits[35]. Their observations were entirely qualitative, but prophetic, in that they suggested that renal nerve stimulation induced redistribution of blood flow from the outer cortex to the inner cortex and medulla. In contrast, Houck (in 1951), who also used the Indian ink distribution method in anaesthetized dogs, to study the effects of intense electrical stimulation of the renal nerves, concluding that CBF and MBF were similarly dramatically decreased by intense renal nerve stimulation[36]. Similar conclusions were drawn by Aukland in 1968, using a method for determining local H_2 gas clearance within the outer medulla in anaesthetized dogs. They found that total RBF and outer cortical H_2 gas clearance both fell by ~40% during intense renal nerve stimulation, but also conceded that 'due to the counter current exchange of gas between ascending and descending vasa recta, the clearance is not necessarily linearly related to blood flow'[37]. Similar observations, using a similar technique in anesthetized rats, were reported by Chapman *et al.* in 1982[38]. Thus, with the exception of the initial study by Trueta *et al.*, the unanimous conclusion from the studies described above was that CBF and MBF are similarly sensitive to the effects of activation of the renal sympathetic nerves.

Some studies were performed in which graded neural stimuli were applied, but the picture arising from them was far from clear. Pomeranz *et al.* (1968) used the ^{85}Kr autoradiography technique in both anaesthetized and conscious dogs, and concluded that although intense renal nerve activity reduced both CBF and MBF, mild stimulation of the renal nerves actually increased MBF[1]. In almost direct contrast, Hermansson *et al.* reported their study using ^{86}Rb uptake in anaesthetized rats in 1984, concluding that MBF was more sensitive than CBF to the ischaemic effects of low frequency renal nerve stimulation[39]. These observations are clearly at odds with the results of more recent studies using laser Doppler flowmetry.

Studies using laser Doppler flowmetry

At present, the most widely used method for estimation of blood flow in specific regions of the kidney is laser Doppler flowmetry. This technique has the advantage that measurements can be made in real-time, and in anatomically specific regions of the kidney. There is good evidence of a direct relationship between laser Doppler flux and erythrocyte velocity both in model systems *in vitro*[15,40-42], and in the kidney *in vivo*[15,40,43,44]. However, it must also be recognised that in highly perfused tissues such

as the kidney, laser Doppler flux is relatively insensitive to changes in the volume fraction of red blood cells in the tissue[15,40,41]. Therefore, changes in MBF due to changes in the number of perfused capillaries (capillary recruitment) are unlikely to be detected by laser Doppler flowmetry. Nevertheless, this method does represent a considerable technical breakthrough in the study of regional kidney blood flow. Over the last decade, studies from a number of separate research groups using this technique have led to the unequivocal conclusion that MBF is relatively insensitive to renal sympathetic drive, especially at stimulus intensities within the physiological range.

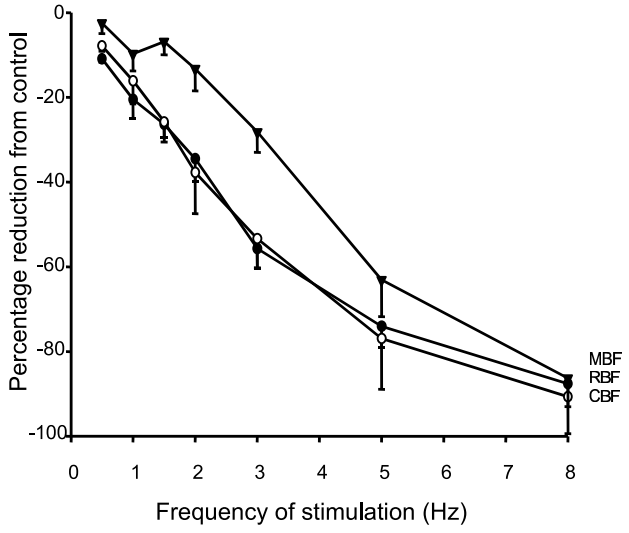

Figure 3. *Mean responses of total renal blood flow (RBF, ●), and laser Doppler flowmetry measurements of cortical blood flow (CBF, ○) and medullary blood flow (MBF,▼), to graded frequencies of renal nerve stimulation (supramaximal voltage, 2 ms duration) in anaesthetized rabbits. Symbols represent mean ± s.e.mean of observations in 8 rabbits. Note that analysis of variance showed that, across all frequencies of electrical stimulation, responses of MBF differed from those of RBF and CBF (*$P < 0.001$*). In contrast, responses of RBF and CBF were indistinguishable (*$P > 0.05$*). Redrawn from Leonard* et al.*[45]*

Rudenstam *et al.* showed that graded renal nerve stimulation (2-5 Hz at 5 V and 1 ms duration) in anaesthetized rats produced progressive reductions in RBF and CBF, but only small changes in blood flow in the renal papilla (the very inner part of the medulla)[2]. We subsequently performed similar studies in anaesthetized rabbits, showing that in this species inner MBF was reduced in a progressive fashion by graded (frequency or amplitude) renal nerve stimulation, but that MBF was reduced less than RBF or CBF, particularly at stimulation frequencies of 3 Hz or less[45] (Figure 3). Collectively, these studies suggested that the medullary circulation is relatively insensitive to the ischaemic effects of renal sympathetic drive, but also raised the possibility that some regional differences in sensitivity might exist within the medulla. To investigate this latter possibility, we tested the effects of graded renal nerve stimulation on laser Doppler blood flow measurements at 2 mm intervals from the surface of the cortex to close to the tip of the papilla[42]. We found that responses to renal nerve stimulation in the renal cortex (≤ 3 mm below the kidney surface) were always greater than those within the medulla (≥ 5 mm below the kidney surface), but that responses within the inner and outer medulla were indistinguishable. Thus, while these data confirm that renal nerve activation can differentially affect CBF and MBF, they do not support the notion that it can differentially affect perfusion at different levels of the medulla.

Impact of endogenous renal sympathetic nerve activity on MBF

Electrical stimulation of the renal sympathetic nerves is a useful technique for producing graded increases in renal sympathetic drive, but it does not mimic naturally occurring renal sympathetic nerve activity (RSNA)[46]. Endogenous RSNA has a bursting pattern, with the amplitude of each burst probably largely reflecting the recruitment of individual axons[47]. In most cases, reflex changes in RSNA mainly reflect changes in the amplitude of bursts, rather than changes in their frequency[48,49]. Relating the frequency of electrical stimulation to changes in endogenous RSNA is therefore problematic[46]. Given this caveat, we can at least say that similar reductions in CBF of ~20% are achieved in anaesthetized rabbits with 1 Hz electrical stimulation[45], and a hypoxic stimulus that increases RSNA by ~80%[50]. Therefore, our observation that the relative insensitivity of MBF to renal nerve stimulation is most clearly seen at low frequencies of stimulation raises the possibility that MBF might be refractory to the basal level of RSNA, and to reflex increases in RSNA associated with physiological manoeuvres that reduce RBF. This does indeed seem to be the case. For example, CBF but not MBF is reduced by arterial chemoreceptor stimulation in conscious rats[51] and hypoxia in anaesthetized rabbits[50] (Figure 4). Furthermore, while hypotensive haemorrhage consistently reduces CBF, MBF has been observed to either remain unchanged or to increase[52,53], or to be reduced less than CBF[54-56]. Conversely, renal denervation in anaesthetized rats increases CBF but not MBF[57].

On the other hand, MBF does not appear to be entirely insensitive to reflex increases in RSNA. Evoking the nasopharyngeal reflex in conscious rabbits, by exposure to cigarette smoke, transiently increased RSNA by ~135%[48]. This reflex is accompanied by little change in arterial pressure, but falls in cardiac output, RBF, CBF and MBF are observed[58] (Figure 5). Indeed, MBF and CBF were reduced similarly by the nasopharyngeal reflex in conscious rabbits, which seems at odds with the notion that MBF is less sensitive than CBF to reflex increases in RSNA. An explanation for this paradox might lie in differences between the dynamic responses of CBF and MBF to neural activation. In particular, MBF seems able to respond faster to renal sympathetic activation[59], and to be

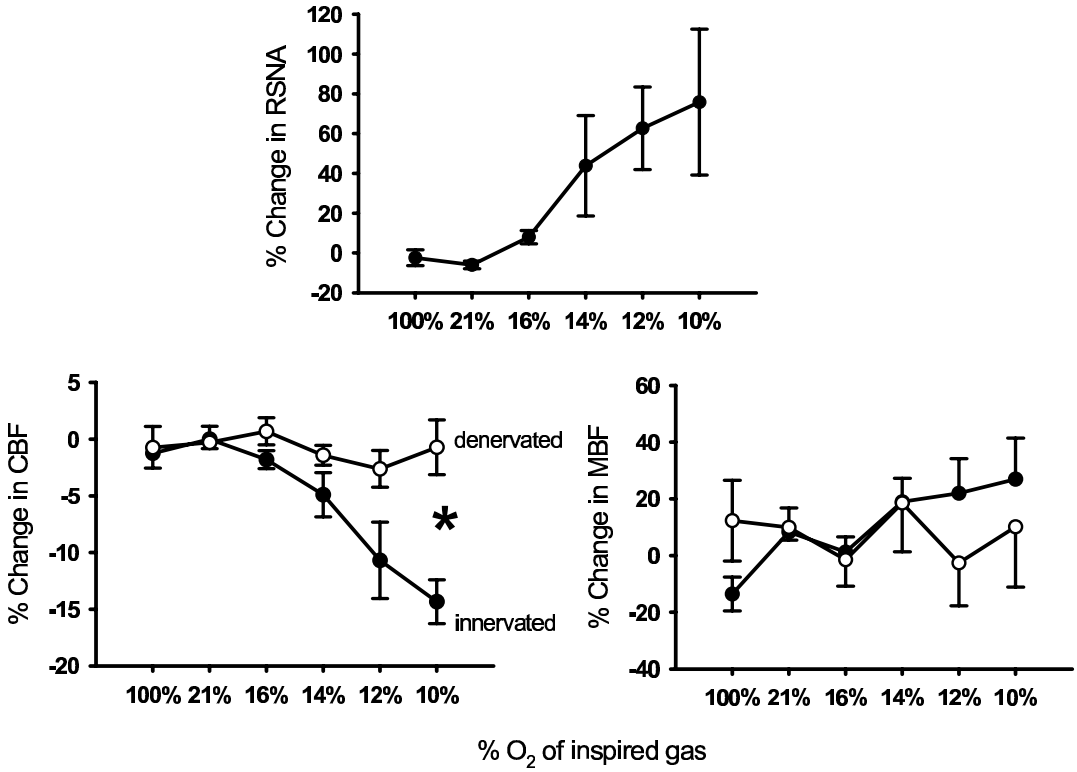

Figure 4. *Responses of renal sympathetic nerve activity, and laser Doppler measurements of cortical blood flow (CBF) and medullary blood flow (MBF) to progressive hypoxia. Experiments were performed in anaesthetized, artificially ventilated rabbits, and hypoxaemia was induced by exposure to progressively hypoxic gas mixtures. Responses of CBF and MBF were determined in rabbits with intact renal nerves (●; n = 7) and in rabbits in which the renal nerves were destroyed (○; n = 6). Symbols and error bars represent mean ± s.e.mean. * P < 0.05 for interaction term between 'state' (intact or denervated) and the response to progressive hypoxia, from analysis of variance. Data redrawn from Leonard* et al.[50]

more sensitive than CBF or total RBF to oscillations in RSNA at frequencies normally present in endogenous RSNA[45]. This might increase the relative responsiveness of MBF to transient increases in RSNA associated with manoeuvres such as the nasopharyngeal reflex. The mechanistic and anatomical bases of the differing frequency response characteristics of CBF and MBF remain unknown.

Mechanisms underlying the relative insensitive of MBF to sympathetic drive

Because MBF is refractory to mild to moderate increases in RSNA, it seems likely that the renal nerves play little role in its physiological regulation. However, in pathological conditions such as heart failure, where RSNA can increase by over 200%[60], MBF might be chronically reduced, which would exacerbate salt and water retention. Furthermore, MBF might also be chronically reduced if its sensitivity to RSNA were somehow increased, perhaps through failure of mechanisms protecting the medulla from the ischaemic effects of sympathetic activation. Potentially, this could lead to the development of hypertension. Much of our recent research, therefore, has focussed on elucidating the mechanisms underlying the relative insensitivity of MBF to renal sympathetic drive. From a theoretical perspective, a number of potential mechanisms could contribute, which are discussed separately below.

Regional heterogeneity of glomerular arteriole geometry

As discussed earlier, (see *The renal medullary circulation: structure and function*), the fact that juxtamedullary afferent and (particularly) efferent arterioles have greater calibre than their counterparts in other regions of the kidney, should theoretically predispose MBF to be less sensitive than the bulk of CBF to virtually all vasoconstrictor stimuli. In support of this notion, we have found that while some vasoconstrictors preferentially reduce MBF more than CBF (eg vasopressin peptides), most reduce CBF more than MBF (eg RSNA, angiotensin II, endothelin peptides)[58,61-67]. Furthermore, renal arterial infusions of angiotensin II[4] and endothelin-1[66] constrict juxtamedullary afferent and efferent arterioles similarly to their counterparts in other regions of the kidney (determined by vascular casting methods), yet MBF is little affected by these agents in the face of large changes in total RBF and CBF[65,66]. It seems likely, therefore, that the vascular architecture of the kidney is arranged in a way that protects the medulla from the ischaemic effects of a range of vasoconstrictor stimuli, including sympathetic nerve activation.

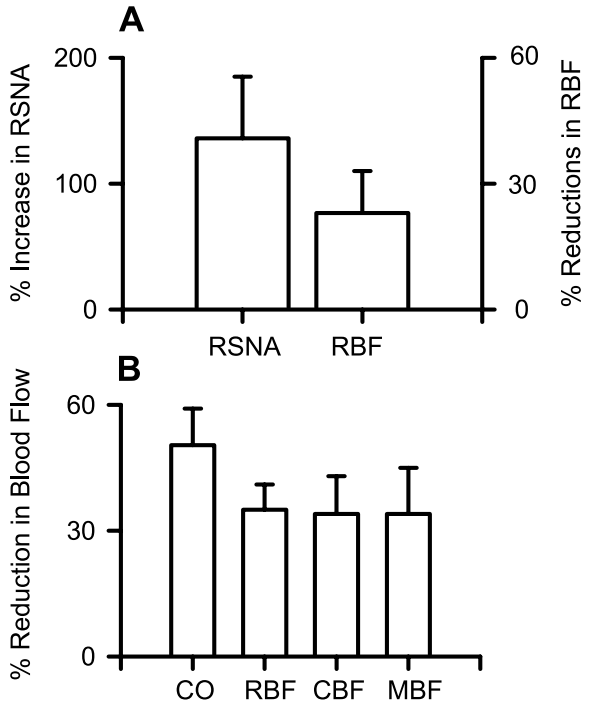

Figure 5. *Responses in conscious rabbits of renal sympathetic nerve activity (RSNA), renal blood flow (RBF), cardiac output (CO), cortical blood flow (CBF) and medullary blood flow (MBF) to exposure to cigarette smoke (the nasopharyngeal reflex). Panel A represents the results of a study in rabbits equipped for simultaneous measurement of RSNA and RBF in the left kidney[48]. Note that changes in RSNA and RBF are shown on different scales. Panel B shows the results of an experiment in rabbits equipped for simultaneous measurement of CO, and RBF, CBF and MBF in the left kidney[58]. The reflex comprises transient reductions in heart rate, CO and RBF that usually reach a maximum within the first 5 s after exposure to smoke. Data represent the mean ± s.e.mean (n = 8-12) of maximum changes from control. Note that responses of RBF in the two experiments are comparable, and that both CBF and MBF are reduced by this reflex which more than doubles RSNA.*

Regional differences in the density of nerve bundles and/or varicocities innervating vascular elements controlling CBF and MBF

As previously mentioned (see *Innervation of vascular elements controlling MBF*), available evidence suggests that juxtamedullary glomerular arterioles and outer medullary descending vasa recta are richly innervated, so this seems unlikely to account for the relative insensitivity of MBF to sympathetic activation. However, more detailed information at the ultrastructural level, regarding the density of neuromuscular junctions on the various vascular elements within the kidney, is required before this potential mechanism can be completely excluded.

Regional differences in sympathetic co-transmitter function in vascular elements controlling CBF and MBF

We recently tested the effects of blockade of α_1-adrenoceptors on regional kidney blood flow responses to renal nerve stimulation[68]. As expected, the α_1-adrenoceptor antagonist prazosin greatly blunted responses of RBF and CBF to renal nerve stimulation, but to our surprise, had no detectable effect on responses of MBF. We can exclude roles for α_2-adrenoceptors in mediating the post-junctional response to renal nerve stimulation, because the α_2-adrenoceptor antagonist rauwolscine did not inhibit responses of MBF to renal nerve stimulation. These observations raise the interesting possibility that sympathetic co-transmitters make an important contribution to mediating the effects of sympathetic nerve activity on MBF.

Interactions between hormonal and neural mediators of renal vascular tone: paracrine hormones

The role of the vascular endothelium in modulating responses to vasoactive factors is well established[10]. More recently, it has become clear that such factors are also released from the tubular epithelium, and that so-called 'tubulovascular cross-talk' plays a key role in the regulation of renal vascular tone[10]. Previous studies of the contribution of these mechanisms to the neural control of regional kidney blood flow have, for the most part, relied on intravascular administration of noradrenaline as a surrogate for neural noradrenaline release. Such experiments must be interpreted with care, since noradrenaline infusion does not adequately mimic sympathetic nerve activation, which likely involves neurotransmitter (including co-transmitter) release at specialised neuromuscular junctions[25]. Nevertheless, these experiments have provided important mechanistic information that has formed the basis of our research in this area.

The relative insensitivity of MBF to noradrenaline infusions (intravenous or renal arterial) appears to be largely due to nitric oxide release[61,69]. Our recent results suggest that a similar mechanism might operate to protect the medulla from the ischaemic effects of sympathetic nerve activation, since blockade of nitric oxide synthesis[62] enhances MBF responses to renal nerve stimulation in rabbits. However, even after nitric oxide synthase blockade, renal nerve stimulation still reduces MBF less than CBF[62], indicating that other mechanisms also contribute to the relative insensitivity of the medullary circulation to sympathetic activation. Prostanoids appear to have little net role in modulating renal vascular responses to activation of the sympathetic nerves, as the cyclooxygenase inhibitor ibuprofen did not significantly affect responses of RBF, CBF or MBF to renal nerve stimulation in anaesthetized rabbits[70]. However, we also recently found that under conditions of prior cyclooxygenase blockade, nitric oxide synthase blockade did not enhance the response of MBF to renal nerve stimulation[70]. These observations contrast directly with those of our previous study under conditions of intact cyclooxygenase activity[62], and raise the intriguing

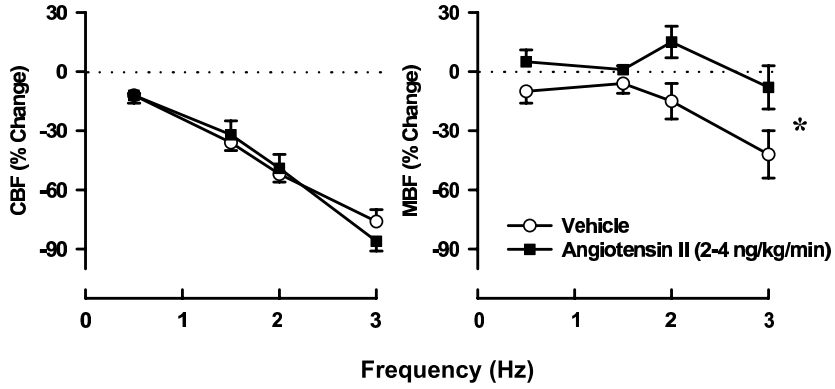

Figure 6. *Responses of cortical blood flow (CBF) and medullary blood flow (MBF) to renal nerve stimulation in anaesthetized rabbits receiving a renal arterial infusion of isotonic saline (○), or angiotensin II (2-4 ng/kg/min, ■) (n = 9). Saline infusion did not significantly affect baseline CBF or MBF, whereas angiotensin II infusion significantly reduced baseline CBF (by 14 ± 5%) but not MBF.* * P < *0.05 for significant difference, across all frequencies, in the responses to renal nerve stimulation during angiotensin II infusion, compared with the responses during saline infusion. Data redrawn from Guild* et al.[63]

possibility, that the impact of nitric oxide synthase blockade on responses of MBF to renal nerve stimulation, are at least partly mediated through vasoconstrictor products of cyclooxygenase.

Interactions between hormonal and neural mediators of renal vascular tone: endocrine hormones

We recently obtained evidence that circulating hormones such as angiotensin II and arginine vasopressin could play a key role in determining the nature of the regional renal haemodynamic response to increased renal sympathetic drive[63]. For example, angiotensin II is known to act at a number of levels to enhance sympathetic neurotransmission[71], but this endocrine/paracrine/autocrine hormone also has a unique action within the medullary circulation, in that it can induce vasodilation through activation of AT_1-receptors, and subsequent release of nitric oxide and vasodilator prostaglandins[10,61,64,65]. To test whether angiotensin II might differentially affect responses to sympathetic activation in the medullary and cortical circulations, we tested the effects, on responses to renal nerve stimulation, of renal arterial infusion of angiotensin II in anaesthetized rabbits, at a dose that slightly reduced basal RBF and CBF but did not significantly affect basal MBF[63]. We found that the angiotensin II infusion virtually abolished reductions in MBF induced by renal nerve stimulation, without affecting responses of RBF and CBF (Figure 6). Thus, elevated intrarenal levels of angiotensin II appear to selectively inhibit renal nerve stimulation-induced ischaemia in the medullary circulation. The physiological significance of this phenomenon, and the mechanisms mediating it, remain to be determined.

Conclusions and future directions

There is now strong evidence that activation of the renal sympathetic nerves has less impact on MBF than CBF, particularly at moderate stimulus intensities. Indeed, the medullary circulation appears to be refractory to basal levels of endogenous sympathetic nerve activity, and to all but the most profound reflex increases in sympathetic drive. The precise nature of the mechanisms that limit the sensitivity of MBF to sympathetic drive remain unknown, although recent experiments suggest roles for nitric oxide and possibly angiotensin II. It also seems likely that regional differences in the geometry of glomerular arterioles pre-disposes MBF to respond less than CBF to any given vasoconstrictor stimulus (Figure 7). Other mechanisms, including the potential for roles of sympathetic co-transmitters, require investigation.

Dysfunction of the mechanisms that protect the medullary circulation from ischaemia due to activation of the renal nerves would increase the sensitivity of MBF to renal sympathetic drive. This could potentially lead to chronic reductions in MBF, salt and water retention, and the subsequent development of hypertension (Figure 7). Future studies should aim to directly test this hypothesis, and determine whether neurally-mediated reductions in MBF contribute to the development of essential hypertension, and also to salt and water retention in pathological conditions associated with increased sympathetic drive, such as heart failure.

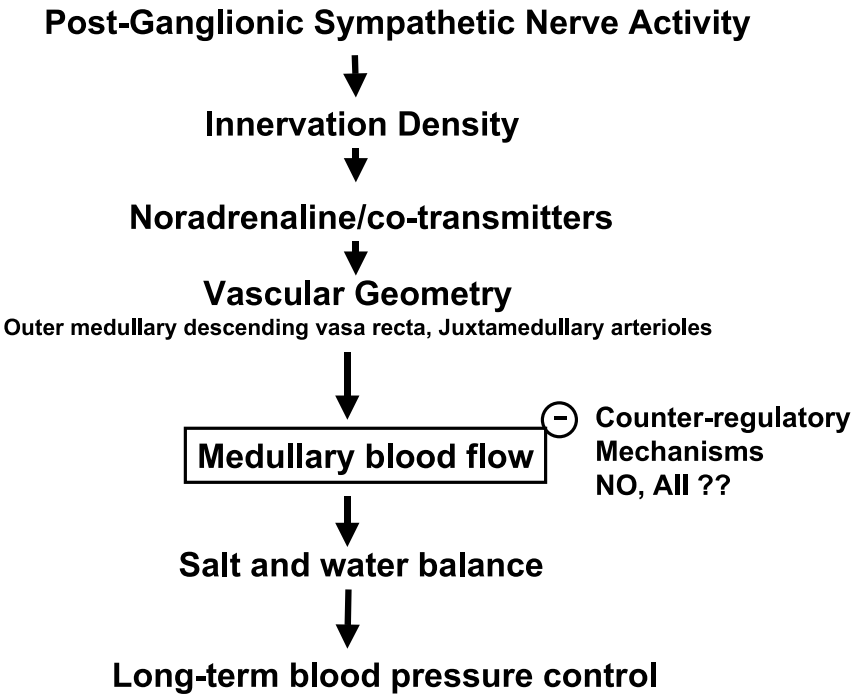

Figure 7. *Working hypothesis of the factors underlying the relative insensitivity of renal medullary blood flow (MBF) to renal sympathetic drive. Responses of MBF to sympathetic activation will depend on the level of post-ganglionic sympathetic nerve activity, the functional density of the sympathetic innervation of vascular elements controlling MBF, on the nature of neurotransmission in these neurones, and on the basal calibre of vascular elements controlling MBF relative to those in the bulk of the renal cortex. Nitric oxide (NO), and perhaps also circulating angiotensin II (AII), seem to play key roles in blunting responses of MBF to renal nerve stimulation. Failure of these mechanisms could lead to salt and water retention under conditions of sympatho-adrenal activation, and so the development of hypertension.*

Acknowledgements

Original studies by the authors described in this article were supported by grants from the National Health and Medical Research Council of Australia (143603, 143785), the National Heart Foundation of Australia (G 98M 0125), the Ramaciotti Foundations (A 6370, RA 159/98), the Marsden Fund of New Zealand, and the Auckland Medical Research Foundation.

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Received 30 October 2003; in revised form January 28 2004. Accepted 29 January 2004.

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