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

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Recently, two new classes of calcium-sensing receptor (CaR) activators have been identified. The type-II calcimimetics (e.g. NPS R-467) were developed from a lead phenylalkylamine compound identified in a large-scale drug screen (Nemeth *et al.*, 1998). Type-II calcimimetics sensitise the CaR to calcium ions by binding to a site in the receptor's transmembrane region (Hauache *et al.*, 2000). 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 (Conigrave *et al.*, 2000). The amino acid binding site is likely to lie in the conserved N-terminal, Venus FlyTrap domain. In the kidney, the CaR is expressed in multiple sites. These include the proximal tubule, the cortical thick ascending limb (CTAL) and the medullary collecting ducts (Ward & Riccardi, 2002). Expression of the CaR in the CTAL has been linked to the control of urinary calcium excretion. Expression of the CaR in the collecting tubule, on the other hand, has been linked to the control of urinary water excretion and osmolality. In particular, CaR activation may suppress 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 (Brown & Hebert, 1996). This pattern of expression implies roles for CaR activators in the regulation of multiple renal functions including proximal tubular transport, calcium excretion and urinary concentration. In particular, CaR-active amino acids (e.g., L-Phe and L-Ala) and type-II calcimimetics are predicted to promote calcium excretion, raise urine flow and suppress urinary osmolality.

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 female Wistar rats (200-300 g), anaesthetised with halothane, both jugular veins were cannulated and the animals were infused (2-4 mL/h) with isotonic physiological saline solution (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, L-amino acids were infused for 60 min prior to return to the control solution. Blood samples (0.25 mL) were collected at regular intervals for analysis of creatinine, osmolality, total calcium and various amino acids. Urine samples were collected at 15 min intervals to assess flow rate, osmolality and creatinine, calcium and amino acids. In some experiments, bolus injections were administered to test for acute effects of R-467 and amino acids. The type-II calcimimetic R-467 enhanced urinary calcium excretion (~3 fold) and urinary flow rate. In addition, R-467 suppressed urinary osmolality consistent with an inhibitory action of the CaR on vasopressin-induced water reabsorption in the collecting ducts. R-467 also lowered serum total calcium levels as previously reported (Fox *et al.*, 1999). The inactive isomer, S-467 was much less effective than R-467 on all three 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, also elevated calcium excretion (~2-fold) and urinary flow rate, and suppressed urinary osmolality. Bolus injections of L-Phe and L-Ala also acutely elevated urinary calcium excretion and flow rate and lowered osmolality.

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.

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Molecular changes in proximal tubule function in diabetes mellitus

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Our studies focus on the changes in the tubulointerstitum (proximal tubule, cortical fibroblast and endothelial cells) that occur when exposed to a 'diabetic environment', i.e. high glucose, low density lipoproteins and cytokines implicated in the pathogenesis of diabtic nephropathy. The current discussion focuses on the specific changes in proximal tubule function that occur following exposure to high glucose. In the normal kidney, the proximal tubule plays a crucial role in the reabsorption of 50-70% of the filtered Na^+ and the receptor mediated uptake of the protein that crosses into the filtrate from the glomerulus. Diabetic nephropathy is frequently associated with increased Na^+ retention, proteinuria and thickening of the tubular basement membrane is the earliest histological abnormality.

Using experimental models, which include both primary cultures of human proximal tubule (hPTC) cells as well as immortalised cell lines, we found initially that high glucose increases the activity of Na^+-H^+ exchanger 3 (NHE3), the key transporter that mediates Na^+ uptake in the proximal tubule. This increase is paralleled by an increase in the mRNA for NHE3. There is also a similar increase in the activity and protein levels of the V-H^+-ATPase, which plays a role in HCO_3^- reabsorption. In a recent study we have shown that the activity of NHE3 is upregulated by exposure to albumin. NHE3 and V-H^+-ATPase are also known to be critical in the endocytosis of albumin, and indeed further studies confirmed that tubular exposure to high glucose increased albumin uptake. These data suggest a possible mechanism linking defective Na^+ reabsorption and protein handling in the kidney in diabetes mellitus.

One of the key regulators of proximal tubule function implicated in the pathogenesis of diabetic nephropathy is angiotensin II (AngII), We have shown that tubular production of AngII is increased significantly following exposure to high glucose. As AngII is also known to increase the activity of both NHE3 and V-H^+-ATPase this may underlie the abnormalities in transport and tubular protein reabsorption in diabetic nephropathy. The profibrotic cytokine transforming growth factor beta (TGFβ) has been shown to be upregulated in animal models of diabetic nephropathy and normalised by blockade of the renin-angiotensin system. We have demonstrated that high glucose induces TGFβ mRNA within 30 minutes of exposure. TGFβ in turn induces both collagen and non-collagen matrix production in the proximal tubule, an effect that is facilitated by the autocrine production of CTGF.

Thus our data provide further insights into the mechanisms by which high glucose induces tubular pathology in the human kidney and are consistent with the deleterious effects of high glucose being mediated at least in part by elevated intrarenal AngII and downstream cytokine production.

Differential neural control of glomerular ultrafiltration

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Homer Smith dismissed the renal nerves in his landmark book The Physiology of the Kidney (1937). Following the first kidney transplantations, the apparent lack of long-term effects on body fluid balance were taken as confirmation of the independence from nervous system control of renal vascular and tubular function. It is now appreciated that transplanted kidneys rapidly re-innervate and that changes in renal nerve activity are implicated in many clinical conditions (Dibona & Kopp, 1997).

The resurgence of interest in the neural control of renal function followed the first comprehensive anatomic study of the renal innervation (Barajas, 1978), demonstrating that all the major structural elements of the kidney were innervated. Although the glomerular afferent and efferent arterioles are densely innervated, the prevailing view today is that tubular innervation is of greater importance for body fluid homeostasis. Reviewing the literature, Dibona & Kopp (1997) argued that the evidence supports the hypothesis that changes in renal nerve activity around resting levels affect renin secretion and tubular function but not blood vessel tone. However, the majority of studies utilised electrical stimulation of the renal nerves, which does not resemble the normal nerve discharge pattern. Our studies suggest a very different situation.

Performing a detailed analysis of the renal innervation, we demonstrated that there are two distinct nerve types, that are differentially distributed to afferent and efferent arterioles (Luff *et al.*, 1992). Type I nerves almost exclusively innervate the afferent arteriole (Luff *et al.*, 1992). Type II nerves, are NPY positive (Anderson *et al.*, 2001) and evenly distributed on both arterioles (Luff *et al.*, 1992). We hypothesised that the different patterns of sympathetic outflow to the kidney may evoke selective changes in glomerular ultrafiltration.

We examined the effects of physiologically induced increases in renal sympathetic nerve activity (RSNA) in response to graded hypoxia on renal pre and postglomerular vascular resistances in anaesthetised rabbits (pentobarbitone, 90-150 mg plus 30-50 mg/h) (Denton *et al.*, 2002b). We demonstrated that 10% oxygen (O_2) caused neurally mediated increases in both pre and postglomerular resistance as reflected by the decrease in both renal blood flow (RBF) and glomerular filtration rate (GFR). However, 14% O_2 which induced a lesser increase in RSNA caused a predominant increase in postglomerular resistance and maintenance of GFR at a time when renal blood flow fell. These results provide evidence that different levels of reflexly induced increases in RSNA may differentially control pre- and post- glomerular vascular resistances, compatible with selective activation of Type I and II renal sympathetic nerves. A caveat to this conclusion was that, though in response to 14% O_2 plasma renin activity was not increased, intrarenal actions of neurally stimulated ANG II may have been responsible for the increase in postglomerular resistance in response to 14% O_2.

This question was investigated in rabbits receiving an ANG II clamp infusion (Denton *et al.*, 2002a). Measurements were made before (room air) and after 14% O_2. As seen in the previous study RSNA increased in response to 14% O_2 and decreased RBF without effecting GFR or arterial pressure. However, glomerular capillary pressure increased in both the vehicle and ANG II clamp groups during 14% O_2, indicating that ANG II was not responsible for the increase in glomerular pressure following RSNA. These results are compatible with our hypothesis that different populations of renal nerves selectively control pre and postglomerular resistance and hence glomerular pressure and ultrafiltration.

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Neural control of renal medullary perfusion

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Over the last decade, evidence has accumulated that the renal medullary circulation plays a key role in regulating arterial pressure in the long-term (Mattson, 2003). A favoured hypothesis rests on the notion of relatively poor autoregulation of medullary blood flow (MBF), allowing changes in MBF in response to changes in arterial pressure to initiate compensatory alterations in tubular sodium reabsorption. Indeed, alterations in MBF have been proposed as the chief mediator of pressure diuresis/natriuresis (Mattson, 2003). We have set out to elucidate the mechanisms that regulate MBF under physiological conditions. A key finding from our studies using laser Doppler flowmetry in anaesthetized (pentobarbitone, 90-150 mg plus 30-50 mg/h) and conscious rabbits, has been that vasoactive hormones can differentially affect MBF and cortical blood flow (CBF) (Evans *et al.*, 2000). This likely represents an important mechanism underlying hormonal control of blood pressure.

Until recently, our understanding of the impact of the renal sympathetic nerves on MBF has been rudimentary. Our recent findings show that MBF is less sensitive than CBF, to electrical stimulation of the renal nerves, particularly at low frequencies of stimulation (Leonard *et al.*, 2000). The responses of MBF to renal nerve stimulation appear to be similar in the outer and inner medulla (Guild *et al.*, 2002). We have also obtained evidence that the medullary circulation is normally insensitive to increases in endogenous renal sympathetic nerve activity within the physiological range, in that increases in renal sympathetic nerve activity of ~80% induced by hypoxia reduce CBF (by ~14%) but not MBF (Leonard *et al.*, 2001).

Our attention has now turned to elucidating the mechanisms underlying the relative insensitivity of MBF to renal nerve activation. Failure of these mechanisms would promote reductions in MBF in response to physiological activation of renal sympathetic nerve activity, which could in turn lead to salt and water retention and hypertension. We have preliminary evidence for a paradoxical role of angiotensin II in selectively blunting responses of MBF to activation of the renal sympathetic nerves. The renal medulla is unique in that, under certain conditions, angiotensin II can induce vasodilatation through release of nitric oxide and/or prostaglandins (Duke *et al.*, 2003). In anaesthetised rabbits, renal arterial infusion of angiotensin II at a dose that reduced basal CBF but not MBF, abolished reductions in MBF induced by renal nerve stimulation (Guild *et al.*, 2003). Ongoing studies are also investigating the roles of nitric oxide, prostaglandins, adrenoceptor subtypes, and sympathetic co-transmitters in the neural regulation of MBF.

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