

## SA14

**THE ROLE OF PURINERGIC RECEPTORS ALONG THE RENAL MICROVASCULATURE.**

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Autoregulation of renal blood flow is an established physiological phenomenon, however the signaling mechanisms involved remain elusive. Autoregulatory adjustments in preglomerular resistance involve myogenic and tubuloglomerular feedback influences. While there is general agreement on the participation of these two regulatory pathways, the signaling molecules and effector mechanisms have not been identified. Currently, there are two major hypotheses being considered to explain the mechanism by which tubuloglomerular feedback signals are transmitted from the macula densa to the afferent arteriole. The adenosine hypothesis proposes that the released ATP is hydrolyzed to adenosine and this product stimulates preglomerular vasoconstriction by activation of A1 receptors on the afferent arteriole. Alternatively, the P2 receptor hypothesis postulates that ATP released from the macula densa directly stimulates afferent arteriolar vasoconstriction by activation of ATP-sensitive P2X1 receptors. This hypothesis has emerged from the realization that P2X1 receptors are heavily expressed along the preglomerular vasculature. Exposure of the renal microvasculature to ATP, or to P2X1 agonists evokes an exclusively preglomerular vasoconstriction that is most prominent in the afferent arteriole. ATP-dependent vasoconstriction and the components of autoregulatory behavior are blocked by L-type calcium channel antagonists. Inactivation of P2X1 receptors impairs autoregulatory responses while afferent arteriolar responses to A1 adenosine receptor activation are retained. Autoregulatory behavior is markedly attenuated in mice lacking P2X1 receptors but responses to adenosine A1 receptor activation remain intact. More recent experiments suggest that P2X1 receptors play an essential role in TGF-dependent vasoconstriction of the afferent arteriole. Interruption of TGF-dependent influences on afferent arteriolar diameter, by papillectomy or furosemide treatment, significantly attenuated pressure-mediated afferent arteriolar vasoconstriction in wild-type mice but had no effect on the response in P2X1 knockout mice. Autoregulation is impaired in hypertension. Assessment of afferent arteriolar responsiveness to P2 receptor stimulation revealed attenuated ATP-mediated afferent arteriolar vasoconstriction in kidneys subjected to 6 and 13 days of angiotensin II-dependent hypertension, compared to normotensive controls. Furthermore, in similarly prepared kidneys, responsiveness to P2X1 receptor stimulation was nearly abolished. Collectively, these observations support an essential role for P2X1 receptors in pressure-mediated autoregulatory behavior and strongly implicate P2X1 receptors in TGF-mediated afferent arteriolar vasoconstriction.

## SA15

**PURINERGIC SUB-TYPES ALONG THE NEPHRON - IMPLICATIONS FOR FUNCTION AND PATHOPHYSIOLOGICAL STATES**

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It is becoming increasingly clear that the extracellular nucleotides ATP, ADP, UTP and UDP can affect solute and water transport in a variety of epithelia, including the nephron. Their actions are mediated by P2 receptors, broadly divided into two types: metabotropic P2Y receptors (mammalian sub-types 1, 2, 4, 6 and 11-14) and ionotropic P2X receptors (mammalian sub-types 1-7). Various P2 receptor sub-types are present in the kidney, both in the vasculature (see previous presentation) and in the tubular epithelium. Until recently, attempts to identify the P2 receptor sub-types in native renal epithelium relied largely on measurements of altered intracellular  $\text{Ca}^{2+}$  concentration in response to application of selected nucleotides, or on determination of mRNA expression (e.g., Bailey *et al.* 2000). The former approach suffers from poor specificity of P2 agonists and from uncertainty as to whether intracellular  $\text{Ca}^{2+}$  transients are a useful index of physiological function (Lehrmann *et al.* 2002); while the latter does not guarantee the presence of receptor protein.

Using immunohistochemical techniques and available polyclonal antibodies, our laboratory has recently identified a number of P2 receptor sub-types along the rat nephron (Turner *et al.* 2003). P2Y<sub>1</sub> and P2Y<sub>4</sub> receptors were found in proximal tubule, and P2Y<sub>2</sub> receptors in both thin and thick ascending limb of Henle, as well as intercalated cells of medullary collecting duct. Low-level expression of P2X<sub>4</sub> and P2X<sub>6</sub> receptors was seen throughout the nephron, while P2X<sub>5</sub> receptors were present in the S3 segment of the proximal tubule and (usually) the collecting duct. Our knowledge of the functional consequences of stimulation of these receptors in native tissue is incomplete. A recent study has shown that stimulation of apical P2Y<sub>1</sub> receptors in rat proximal tubule can inhibit bicarbonate reabsorption (Bailey, 2004), but no information is yet available concerning the functional response (other than  $\text{Ca}^{2+}$  transients) to P2 receptor stimulation in the loop of Henle or distal tubule. Most attention has focussed on the collecting duct. It has been known for some time that basolateral P2 receptor stimulation inhibits vasopressin-stimulated water reabsorption in collecting ducts. More recently, it has been shown that stimulation of apical P2 receptors in collecting ducts inhibits  $\text{K}^{+}$  channels and ENaC-mediated sodium reabsorption (Unwin *et al.* 2003). However, the receptor sub-types responsible for these effects have not been firmly established: pharmacological profiling suggests that inhibition of sodium reabsorption in mouse collecting duct *in vitro* is mediated by P2Y<sub>2</sub> receptors (Lehrmann *et al.* 2002), whereas our own *in vitro* studies in the rat point towards a P2X-mediated effect, possibly a P2X<sub>4/6</sub> heteromer.

Both P2Y and P2X receptors have also been shown to influence cell proliferation and differentiation, as well as cell death by necrosis and apoptosis. These effects might be important in some pathological conditions such as polycystic kidney disease, in which 'trapped' ATP could promote cyst development and expansion by stimulating cell proliferation and fluid secretion; and in

some forms of renal inflammation, in which ATP released as a result of vascular injury and thrombosis may itself cause further cell injury and loss, as well as release of pro-inflammatory cytokines.

Finally, it should be emphasized that, despite the physiological and pathological possibilities outlined above, we have only limited direct evidence for a regulatory role of this receptor system in the kidney. However, in our view this almost certainly reflects the complexity of the system, as well as our currently limited means of investigating it.

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Lehrmann H *et al.* (2002). *J Am Soc Nephrol* 13, 10-18.

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## SA16

### Adenosine as a Paracrine Regulator of Nephron Function

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The actions of adenosine are mediated by 4 types of G protein-coupled membrane receptors, A1, A2a, A2b, and A3 adenosine receptors (A1AR, A2aAR, A2bAR, A3AR). All types of adenosine receptors are expressed in the kidney, both in vascular and epithelial cells. We have focused on the role of adenosine 1 receptors (A1AR) which are predominantly found in afferent arterioles and at lower expression levels in proximal tubules and other nephron segments.

Adenosine causes a long-lasting and dose-dependent vasoconstriction of afferent arterioles that is most pronounced in the terminal portion of the vessel. In perfused mouse afferent arterioles adenosine-induced vasoconstriction is caused by Gi-protein-dependent activation of PLC and subsequent release of Ca from SR stores through an IP3-dependent pathway. Vasoconstriction is maintained by activation of Ca-activated Cl channels, depolarization, and Ca influx through voltage-dependent Ca channels. To cause afferent arteriolar vasoconstriction, adenosine had to be added to the bath. Inclusion of adenosine in the perfusate caused vasoconstriction only after inhibition of NO synthases indicating that intravascular adenosine leads to the release of endothelial dilators that counteract the A1AR-mediated smooth muscle cell activation. Studies in A1AR knockout mice have shown that the vasoconstriction that is normally elicited by an increase in NaCl concentration at the macula densa site of the nephron (tubuloglomerular feedback, TGF) is completely absent in A1AR-deficient animals suggesting that adenosine is a critical component of the TGF signaling pathway. Fluid and electrolyte absorption along proximal tubules and loops of Henle were largely normal in A1AR<sup>-/-</sup> mice in contrast to the inhibitory effect of acute administration of A1AR antagonists on proximal fluid reabsorption. Thus, while the role of adenosine in TGF is non-redundant, its effect on proximal transport is fully compensated during chronic A1AR deficiency. The recent demonstration of NaCl-dependent ATP release by MD cells (Bell

*et al.* PNAS 2003) raises the possibility that adenosine in the juxtaglomerular interstitium may be formed from nucleotide precursors by the action of ecto-ATPases with the final step from 5-AMP to adenosine being mediated by ecto-5-nucleotidase/CD73 (e-5-NT/CD73). In order to study the possible role of this pathway in TGF, we generated e-5-NT/CD73-deficient mice by gene targeting. Wild type mice, and mice heterozygous and homozygous for the e-5-NT/CD73 null mutation were born in a near Mendelian ratio. E-5-NT/CD73<sup>-/-</sup> mice were viable and showed no gross anatomical abnormalities. RT-PCR and immunohistochemistry confirmed the absence of e-5-NT/CD73 mRNA transcripts and protein in the kidneys of e-5-NT/CD73 knockout mice. Blood pressure measured by the tail cuff method and blood chemistry with the exception of a significant increase in alkaline phosphatase appeared normal. E-5-NT/CD73<sup>-/-</sup> mice showed a marked decrease in the bradycardic response of i.v. injected 5-AMP, an effect that is mediated by adenosine and absent in A1AR<sup>-/-</sup> mice.

Micropuncture experiments to address TGF function showed that e-5-NT/CD73<sup>-/-</sup> mice had a significantly reduced change in stop flow pressure (Psf) to an increase of tubular perfusion flow from 0 to 30 nl/min of  $-5.5 \pm 0.8$  mmHg (n=24) compared to their wild type littermates ( $-9.8 \pm 0.9$  mmHg, n=13, p=.002). Similarly, the fall of early proximal flow rate to the same saturating loop flow increase was significantly reduced ( $-5.3 \pm 0.5$  nl/min in wild type, and  $-1.6 \pm 0.5$  nl/min in e-5-NT/CD73<sup>-/-</sup>; p<.0001). Furthermore, whereas TGF responses did not change significantly during repeated changes in perfusion flow (up to 5 times) in e-5-NT/CD73<sup>+/+</sup> mice, a gradual decline in the magnitude of the residual TGF response was noted in e-5-NT/CD73<sup>-/-</sup> mice with TGF responses falling from the initial decrease of  $-5.5$  mmHg (n=24) to only  $-1.3 \pm 0.5$  mmHg during the fifth response (n=10, p=.004 compared to first response; p<.0001 compared to wild types). These data are in agreement with earlier pharmacological evidence supporting a role of e-5-NT/CD73 in TGF (Thomson *et al.*, JCI 2000). Preliminary observations in P2X1 knockout mice indicate that the presence of this particular P2 receptor subtype is not necessary for TGF responsiveness. We conclude that adenosine has a unique vasoconstrictor role in the kidney that is the result of a highly selective expression of A1AR in a small portion of the afferent arteriole. These receptors are activated in a paracrine fashion by adenosine that is generated in the juxtaglomerular interstitial space and reaches its binding sites from the outside of the vessel. The formation of adenosine in the JG interstitium depends to a large extent on e-5-NT/CD73-mediated hydrolysis of 5-AMP that is presumably generated from released ATP.

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## SA17

### ROLE OF ATP IN THE MACULA DENSA

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The macula densa is a unique group of cells that are located within the thick ascending limb at the point where this segment of the loop of Henle comes in contact with its own glomerulus. Macula densa cells detect changes in tubular fluid sodium chloride concentration ([NaCl]) and then transmit information to the underlying mesangial cell/afferent arteriolar complex. This process is called tubuloglomerular feedback and serves to cou-

ple tubular flow and ion composition with the regulation of glomerular filtration and blood flow. To date, the nature of the signaling process between macula densa cells and the mesangial cell/afferent arteriolar complex has remained elusive. However, recent studies have now suggested that macula densa cells signal through the release of ATP.

NaCl entry into macula densa cells occurs via a Na:2Cl:K cotransporter, thereby leading to a number of cellular responses including elevations in cytosolic [NaCl], basolateral membrane depolarization, changes in cell volume, and increases in intracellular pH and calcium. In contrast to most other cells, intracellular [Na] is removed from macula densa cells via an apically located colonic form of the H:K ATPase. Since the basolateral area of macula densa cells contains a large abundance of mitochondria and very little Na:K ATPase activity, it is possible that this area serves as a reservoir or sink for ATP. In recent patch clamp studies, a large maxi-anion channel has been found at the basolateral membrane of macula densa cells. It has a conductance of ~380 pS and is blocked by Gd<sup>3+</sup>. Using inside-out patches, this maxi-anion channel was shown to be permeable to ATP. At the present time, the molecular identity of this channel remains unknown. In additional studies, a biosensor assay was developed using either PC12 cells or cultured mesangial cells loaded with fura-2 and placed at the basolateral membrane of macula densa cells. ATP release across the basolateral membrane was measured to be in the range of 5 to 10  $\mu$ M. This is well within the range of ATP concentrations that will activate purinergic receptor mediated changes in cytosolic calcium concentration in mesangial cells. Importantly, ATP release was consistent with macula densa cell signaling, including the fact that ATP release was abolished by loop diuretics that inhibit the apical Na:2Cl:K cotransporter. Also, prior salt deprivation, which has been shown to up-regulate macula densa cell signaling likewise caused enhanced release of ATP release.

Other work has utilized a mouse cell culture line that has some of the properties of macula densa cells (from J. Schnermann, NIH). Cells were grown on permeable supports and basolateral release of ATP was detected with a luminometer using the luciferase/luciferin reaction. ATP was released by these cells and could also be inhibited by furosemide or Gd<sup>3+</sup>. One candidate for the maxi-anion channel in macula densa cells is the voltage dependent anion channel (VDAC, or porin), which serves as a mitochondrial ATP channel. VDAC and the MD maxi-anion channel have similar electrophysiological characteristics, and recent work has identified a plasma membrane isoform of VDAC (pVDAC). In the macula densa cell line mRNA was isolated and used in RT-PCR to identify mitochondrial VDAC's (VDAC1, 2, 5). Importantly, the mRNA for pVDAC was also present in this macula densa like cell line. These studies indicate that macula densa cells transmit information through the release of ATP via a maxi-anion channel and purinergic signaling.

harmful in a number of disease states by inducing fibrosis and vascular dysfunction. Novel results from several laboratories suggest that aldosterone also has beneficial effects in the cardiovascular system by stimulating the production of nitric oxide from the endothelium.

We tested the effect of aldosterone on vascular function in microdissected, perfused rabbit renal afferent arterioles. Aldosterone per se had no effect on vascular internal diameter in concentrations from 10<sup>-10</sup>-10<sup>-5</sup> mol/L, but after exposure to aldosterone the ability of 100 mM KCl to induce vascular contraction was abolished. This inhibitory effect of aldosterone was observed from less than 1 picomole/L. The inhibitory effect was significant after 5 min and maximal after 20 min, it was fully reversible, and it was abolished by inhibition of mineralocorticoid receptor with spironolactone (10<sup>-7</sup> mol/L), but not by inhibition of the glucocorticoid receptor with mifepristone. The effect of aldosterone on vasoconstriction was not blocked by inhibition of transcription by Actinomycin D (10<sup>-6</sup> mol/L). Expression of mineralocorticoid receptors and 11-beta-hydroxy-steroid-dehydrogenase 2 was demonstrated by RT-PCR and immunohistochemistry on rat preglomerular renal vasculature and cultures of rat renal vascular smooth muscle cells. Aldosterone did not affect depolarisation-mediated increases in calcium concentration in non-perfused rabbit afferent arterioles as measured by fluorescence-microscopy with Fura-2. Inhibition of phosphatidylinositol (PI)-3 kinase with LY 294002 (3 x 10<sup>-6</sup> mol/L) restored sensitivity to K<sup>+</sup> in the presence of aldosterone and the catalytic PI-3 kinase subunit p110 $\gamma$  was demonstrated in afferent arterioles by immunohistochemistry. Inhibition of nitric oxide formation by L-NAME (10<sup>-4</sup> mol/L) or inhibition of the NO-target in vascular smooth muscle, the soluble guanylyl cyclase, with ODQ also restored K<sup>+</sup>-induced vasoreactivity in the presence of aldosterone. Inhibition of heat shock protein 90 with geldanamycin blocked the effect of aldosterone. As a further indication of involvement of the NO pathway, the effect of the NO-donor sodium nitroprusside was similar to that of aldosterone.

Liu et al. (2003) made similar observations using a different vascular preparation from a different species, and with different agonists. They used rat aortic rings which were mounted in a static setup and precontracted with phenylephrine. Incubation of rings for 2 minutes with aldosterone led to a concentration-dependent vasodilation, which was significant at 10<sup>-12</sup> M aldosterone and maximal at 10<sup>-11</sup> M. and which was blocked by spironolactone and LY 290002. These authors also reported a doubling of PI-3 kinase activity in bovine aortic endothelial cells after exposure to aldosterone, as well as an increase in NO-formation as measured by fluorescence microscopy with DAF fluorophore. We conclude from these results that aldosterone inhibits vasoconstriction by a rapid non-genomic effect. The effect is mediated by the classical mineralocorticoid receptor, and it involves heat shock protein 90, phosphatidylinositol (PI)-3 kinase, endothelial nitric oxide synthase, and liberation of nitric oxide. Based on the results we suggest that healthy individuals with a functioning endothelium the detrimental effects of aldosterone on cardiovascular function are balanced by activation of the potentially beneficial effect of NO. On the other hand, in situations with endothelial dysfunction, such as congestive heart failure and hypertension, the negative effects of aldosterone are unopposed and inhibition of aldosterone may be warranted.

Uhrenholt, TR, Schjerning, J, Hansen, PB, Norregaard R, Jensen BL, Sorensen GL, Skott, O. 2003. Rapid inhibition of vasoconstriction in renal afferent arterioles by aldosterone. *Circ Res* 93, 1258-1266.

## SA18

### NO AND ALDOSTERONE INTERACTIONS IN THE RENAL MICROVASCULATURE

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The main role of aldosterone is to maintain body sodium homeostasis by promoting sodium reabsorption in the collecting ducts of the kidney. In the cardiovascular system aldosterone may be

Liu, S.L., Schmuck, S., Chorazczyewski, J.Z., Gros, R. & Feldman, R.D. 2003. Aldosterone regulates vascular reactivity: short-term effects mediated by phosphatidylinositol 3-kinase-dependent nitric oxide synthase activation. *Circulation* 108, 2400-2406.

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## SA19

### NO AND NEPHRON FUNCTION

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Nitric oxide in the juxtaglomerular apparatus plays an important role in controlling the tubuloglomerular feedback mechanism (TGF) and renin release. A continuous production of NO in the macula densa cells is necessary to reduce the sensitivity of the TGF to prevent fluid volume retention and an overproduc-

tion of renin. In situations where macula densa cell NO concentration is reduced, fluid volume retention occurs and hypertension develops. This occurs in rats long-term treated with the nNOS inhibitor 7NI and also in rats that spontaneously develop hypertension such as the Milan Hypertensive strain (MHS) or spontaneously hypertensive rats (SHR). In particular, macula densa cell NO is an important factor to control the sensitivity of the TGF in a situation with volume expansion when renin production is reduced. In volume expansion TGF sensitivity is reduced to a low level but can be normalized or even sensitised during treatment with 7NI leading to a reduced GFR.

In conclusion the actual NO concentration in the macula densa cells is an important factor to control the sensitivity of the TGF mechanism and also for the release of renin. Decreased NO concentration acts to increase TGF sensitivity leading to decreased GFR and fluid volume excretion and in addition to an increased renin release. Macula densa cell NO concentration is therefore an important factor to determine arterial blood pressure through changes in fluid volume balance.