P2X7 receptor antagonism improves renal blood flow and oxygenation in angiotensin-II infused rats

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ABSTRACT

Chronic activation of the renin angiotensin system promotes hypertension, renal microvascular dysfunction, tissue hypoxia and inflammation. We found previously that the injurious response to excess angiotensin II (ANGII) is greater in F344 rats, whereas Lewis rats are renoprotected, despite similar hypertension. We further identified *p2rx7,* encoding the P2X7 receptor (P2X7R), as a candidate gene for differential susceptibility and here we have tested the hypothesis that activation of P2X7R promotes vascular dysfunction under high ANGII tone.

14-day infusion of ANGII at 30ng/min into F344 rats increased blood pressure by ~15mmHg without inducing fibrosis or albuminuria. *In vivo* pressure natriuresis was suppressed, medullary perfusion reduced by ~50% and the cortico-medullary oxygenation gradient disrupted. Selective P2X7R antagonism restored pressure natriuresis, promoting a significant leftward shift in the intercept and increasing the slope. Sodium excretion was increased 6 fold and blood pressure normalized. The specific P2X7R antagonist AZ11657312 increased renal medullary perfusion, but only in ANGII-treated rats. Tissue oxygenation was improved by P2X7R blockade, particularly in poorly oxygenated regions of the kidney. Activation of P2X7R induces microvascular dysfunction and regional hypoxia when ANGII is elevated. These pro-inflammatory effects may contribute to progression of renal injury induced by chronic ANGII.

Key words: kidney injury, kidney disease, ATP, purinergic, BOLD MRI

INTRODUCTION

Chronic kidney disease (CKD) affects ~10% of the world's population[1] and increases the risk of serious cardiovascular events[2]. The etiology of CKD is varied and its pathophysiology complex. Renal hypoxia due to reduced perfusion of the medulla[3, 4] may be a common underlying early event, triggered or exacerbated by inflammation[5, 6].

CKD is heritable but identifying candidate genes for complex diseases in a heterogeneous human population is challenging[7]. Rodent models replicating key aspects of CKD provide a powerful tool to help identify genetic contributions to injury.[8] In that context the *Ren2* transgenic rat[9] has provided a platform for examining the pathways associated with malignant hypertensive end-organ injury, which has been documented in an extensive literature.[10] Refining the approach by placing *Ren2* cDNA expression under the control of an inducible cytochrome P450 promoter, allowed dose-dependent activation of the renin-angiotensin system by dietary administration of a non-toxic xenobiotic.[11] This improved experimental control permits the sequence of events leading to renal injury to be defined more precisely: in the early phase, renal hemodynamics are stable, tubular sodium transport is increased and injury is localized predominantly to the pre-glomerular arteries and renal microvasculature[12].

In both the constitutive and inducible forms of this model the extent of injury is strongly influenced by genetic background: F344 rats are prone to injury; the Lewis strain background confers renoprotection.[13, 14] We exploited the differential susceptibility of these inbred strains to map Quantitative Trait Loci (QTL) for malignant hypertension and its sequela[13, 15]. One QTL was then captured in

reciprocal congenic lines to identify *Ace* (encoding angiotensin converting enzyme) as a major modifier gene for renal injury[14]. The circulating and local concentration of angiotensin II (ANGII) is increasingly recognized as an important factor in CKD[15].

In the *cyp1a1*-Ren2 rat, blockade of AT1 receptors prevents hypertension, but is only partially protective against renal vascular injury[12] and it is likely that multiple pathways contribute to injury. Therefore we re-mined an exon microarray[14] to compare renal gene expression in non-hypertensive, non-injured F344 and Lewis rats. A bioinformatics enrichment approach confirmed *Ace* as the top ranked candidate gene, but also identified *P2rx7*, which encodes the purinergic P2X7 receptor (P2X7R), as the next leading candidate gene for susceptibility to hypertensive renal injury.[16]

Purinergic signaling exerts powerful physiological effects on renal function[17] and the hypothesis that sustained activation of P2X7R contributes to hypertensive renal vascular injury and dysfunction is attractive. Several studies indicate that P2X7R activation contributes to chronic inflammatory disorders: activation of P2X7R promotes the formation of the NLRP3 inflammasone[18] and stimulates release of inflammatory cytokines[19]. Moreover, P2X7R expression is increased in human and experimental kidney disease[20]. Pharmacological blockade or genetic deletion of this receptor protects against antibody-mediated glomerular inflammation[21] and hypertensive renal injury[22] in rodents. Renoprotection is associated with reduced numbers of renal macrophages and has been attributed to a direct anti-inflammatory effect of P2XR7 antagonism.

However, *P2rx7* mRNA is abundantly expressed under normal, non-inflammatory, conditions on endothelial cells,[23, 24] and the receptor is immunolocalized to the

endothelium of the pre-glomerular vasculature.[16] P2X7R is also expressed in the *vasa recta*[25] and functional data indicate that receptor activation causes contraction of renal medullary pericytes.[26] Importantly, the interstitial concentration of extracellular ATP, the endogenous ligand for P2X7R, is markedly increased by elevated renal perfusion pressure[27] and by chronic infusion of ANGII.[28] This lead us to propose a novel and alternative hypothesis in which the early injurious event of sustained P2X7R activation is vascular in origin: reduced perfusion of the renal medulla would promote hypoxia[29] and impair blood pressure control through a suppressed pressure-natriuresis response.[30]

Therefore, we determined the effect of P2X7R antagonism on the pressure-natriuresis and renal hemodynamic responses in F344 rats chronically infused with ANGII. Blood Oxygenation Level-Dependent magnetic resonance imaging (BOLD MRI) was used to assess oxygenation in the renal cortex and medulla. Our results suggest that tonic P2X7R activation contributes to renal vasoconstriction in rats with high ANGII tone. Blockade of the receptor increases renal oxygenation and P2XR7 inhibitors may have a therapeutic benefit in addition to their known anti-inflammatory actions.

RESULTS

P2X7R antagonism improves the pressure-natriuresis response in ANGII-treated rats

After 14 days of ANGII infusion, mean blood pressure (MBP) was increased by ~15mmHg compared with vehicle infused rats (Figure 1A). Sodium excretion measured under anesthesia was not different from control rats (Figure 1B) but chronic ANGII significantly ($P<0.01$) suppressed the elevation (y-intercept) of the pressure-natriuresis (Figure 1C) and pressure-diuresis (Figure 1D) relationships, as shown previously.[31]

Acute infusion of the selective P2X7R antagonist Brilliant Blue G (BBG)[32] reduced blood pressure in ANGII treated rats to a level similar to control animals (Figure 1A); sodium excretion was increased ~6-fold by BBG (Figure 1B). BBG also caused a significant ($P<0.05$) leftward shift in both pressure-natriuresis and pressure-diuresis curves and the gradient of response was significantly improved ($P<0.01$) compared with ANGII-treated rats receiving vehicle (Figure 1C/D). Renal blood flow, measured by a Doppler flow probe around the left renal artery was not affected by increased blood pressure in any of the experimental groups; glomerular filtration rate was also unchanged during pressure-natriuresis (Supplemental Figure 1).

Chronic ANGII treatment and P2X7R expression

In control rats P2X7R expression was localized to the vascular endothelium of the preglomerular arteries, such as the segmental (Figure 2 A/B) and interlobar arteries (Figure 2C/D). P2X7R staining was also noted in the smooth muscle of the afferent arteriole (Figure 2E/F). In control rats there were low levels of glomerular and tubular expression of P2X7R (Figure 2E), which became more evident after chronic ANGII

treatment (Figure 2F). ANGII also induced focal expression in the vascular smooth muscle of the pre-glomerular arteries (Figure 2Bi). However, at the whole kidney level, mRNA and protein expression of P2X7R was unaffected by ANGII infusion (Supplemental Figure 2). As anticipated, 14 days of ANGII infusion did not induce gross renal injury, there was no evidence of fibrosis, quantified by Sirius red staining, and there was no albuminuria (data not shown). ANGII infusion can promote vascular hypertrophy, but here the cross sectional vessel wall area was not different between groups. ANGII did, however, induce vacuolization of pre-glomerular arteries (Figure 3 A/B). This smooth muscle injury was significantly more extensive in ANG II treated rats than controls (Figure 3C/D) and is suggestive of vasospasm. ANGII infusion also caused glomerular accumulation of ED-1 positive macrophages (Figure 3E), suggesting some inflammatory cell infiltrate after 2 weeks of treatment.

P2X7R antagonism increases renal medullary perfusion in ANGII-treated rats

The effect of P2X7R antagonism on renal and intrarenal blood flow was measured in a separate group of rats infused with ANGII or vehicle for 14 days. ANGII again caused a modest increase in MBP (ANGII= 133±2; Vehicle= 118±2 mmHg; $P<0.01$) and a reduction in heart rate (ANGII 380±4; Vehicle 406±13 bpm; $P<0.05$) but left renal artery blood flow (ANGII= 6.64±0.5; Vehicle= 6.24±0.44 ml/min) and renal vascular resistance (ANGII= 21.0±1.9; Vehicle= 19.2±1.1 mmHg/ml.min^{-1}) were not affected. Medullary microvascular perfusion was reduced by ~50% in ANGII-treated rats (ANGII= 6.5±2.7; Vehicle= 12.02±3.5 TPU; $P<0.05$). After these baseline recordings, the effect of a specific and potent P2X7R antagonist AZ11657312, [33] was assessed. Exemplar recordings of AZ11657312 on renal hemodynamics in an ANGII-treated rat are shown in Supplemental Figure 3; group mean data were obtained by

normalizing the area under the response curve in each rat to changes induced by equivalent volume injections of the vehicle (1% DMSO in saline). Two-way ANOVA indicated a significant overall effect of AZ11657312 ($P<0.001$) on MBP, renal artery blood flow and medullary perfusion. In control rats, IV injection of AZ11657312 caused a reduction in MABP (Figure 4A) and an increase in renal blood flow compared with vehicle, but only at the highest dose (Figure 4B). The effect of AZ11657312 was not significantly greater from that of vehicle at any dose used (Figure 4C). In ANGII-infused rats AZ11657312 induced a dose-dependent reduction of MABP (Figure 4A), accompanied by a dose-dependent increase in both renal blood flow and medullary perfusion (Figure 4B & C).

The renal hemodynamic effect of P2X7R antagonism is nitric oxide-dependent

To test whether the vasoactive effects of P2X7R blockade were dependent on nitric oxide (NO) production, we injected the nitric oxide synthase (NOS) inhibitor, Nω-Nitro-L-arginine methyl ester hydrochloride (L-NAME) into ANGII-treated rats prior to administration AZ11657312. In control rats, the modest hemodynamic effects of AZ11657312 were not significantly affected by NOS inhibition (Supplemental Figure 4).

In the ANGII-treated rats, L-NAME did not change the hypotensive effect of AZ11657312 (Figure 5A) and therefore the blood pressure response to systemic P2X7R antagonism is independent of NO production. L-NAME reduced, but did not abolish, the effect of AZ11657312 on renal artery blood flow (Figure 5B) and medullary perfusion (Figure 5C). This indicates that the vasodilation of the renal vasculature induced by P2X7R antagonism is only partially dependent on NO production.

P2X7R antagonism increases renal oxygenation in ANGII-treated rats

We used BOLD MRI to assess the effect of P2X7R antagonism on the renal map of R2*, which is inversely related to tissue pO_2. An anatomically unbiased k-means clustering approach was used for post-acquisition analysis, because this approach identifies regions sharing quantitative "nearness" or "similarity" regardless of their underlying spatial location, and is not subject to bias that can be associated with user-led analysis.[34] Moreover, this approach includes all of the data within the image to provide a global map of oxygenation homogeneity within the kidney of each rat. [34]

In control rats, the renal R2* map segregated two clusters of homogeneity that largely corresponded to discrete anatomical regions of the kidney. The low R2* cluster (high oxygenation) was mainly situated in the cortex; the high R2* cluster (low oxygenation) localized primarily to the medulla. Injection of the P2X7R antagonist BBG did not affect the R2* signal within either of these clusters (Figure 6A and B).

Chronic ANGII infusion markedly altered the R2* map within the kidney, as we have reported before[34]. Two clusters were segregated since divergence is assured using this algorithm. However, these clusters of high and low R2* did not overlay onto discrete anatomical regions of the kidney, indicating dissipation of the cortico-medullary oxygenation gradient. In this setting, acute P2X7R antagonism produced a significant fall in R2* in both clusters (ANOVA $P<0.01$), reflecting <u>increased</u> oxygenation. This effect was particularly evident in the cluster with low baseline pO_2 levels, in which *post-hoc* comparisons also reached statistical significance (Figure 6B).

DISCUSSION

Increased P2X7R expression is associated with renal injury in humans[20] and experimental models of type I diabetes, hypertension,[22, 35, 36] and glomerular disease[21.] This receptor is highly expressed in immune cells, playing a key role in the activation of macrophages, T- and B- lymphocytes.[37] Injured cells also express the receptor, which in this setting is usually pro-apoptotic or cytotoxic.[21, 38] Not surprisingly, P2X7R is an attractive therapeutic target to reduce inflammation in CKD.[39] Our data suggest that additional benefit may accrue from blockade of renal P2X7R distinct from a direct anti-inflammatory effect: antagonism of the receptor in hypertensive rats reduced renal vascular resistance, consistent with expression in pre-glomerular arteries and arterioles. P2X7R antagonism also increased both perfusion and oxygenation of the medulla, which are beneficial outcomes in CKD.[29] P2X7R blockade additionally improved the pressure-natriuresis response in hypertensive rats, which is an important blood pressure regulating mechanism.[40] The interpretation of these data rests on the selectivity of the P2X7R antagonists, BBG and AZ11657312. BBG is a potent inhibitor of rat P2X7R (IC_{50} = 10 nM) but can also block the closely related P2X4R, albeit with 1000-fold lower selectivity. Non-purinergic off-target effects have also been reported[41] and we cannot exclude the possibility that P2X7R-independent effects contribute to the hemodynamic actions of BBG observed here. Nevertheless, the specific P2X7R inhibitor AZ11657312[33], which does not inhibit P2X4R, evoked vasoactive effects qualitatively similar to those induced by BBG and together our findings strongly suggest that P2X7R activation can influence renal hemodynamic function.

P2X7R is expressed in vascular endothelial cells[23, 24] and in the normal rat kidney it has been immunolocalized to the endothelium of the pre-glomerular arteries and the afferent arteriole.[16] Functional studies are limited, but P2X7R may form cation channels in the endothelium[42] and on activation induce NO production.[43] P2X4R activation may also contribute to increase NO formation.[44] P2X7R can also influence vascular reactivity indirectly by promoting the release of cytokines, particularly IL-1β, from endothelial cells, dependent on inducible NO synthase (iNOS) activation.[45]

The current study in rats with high ANGII tone suggests that P2X7R activation exerts a tonic vasoconstriction of the renal artery and medullary microcirculation. This may contribute to the renal vasodilation induced by PPADS in ANGII rats.[46]. PPADS is a broad-spectrum P2 receptor antagonist but has high affinity at the rat P2X7R. The vasodilatation following P2X7R antagonism was partially dependent on NO formation by NOS. It is also possible that this vasodilatation reflects inhibition of the receptor in the smooth muscle, rather than the endothelium. Smooth muscle expression is normally low[47], but some focal P2X7R immunoreactivity was observed in the present study after chronic ANGII infusion, in keeping with previous data from the hypertensive Ren2 transgenic rat.[36]

The pressure-natriuresis response describes the mechanism by which increased renal perfusion pressure induces an increase in sodium excretion. In the current study, increased sodium excretion largely reflects inhibition of tubular sodium reabsorption, since renal blood flow and GFR were effectively auto-regulated during the applied increases in blood pressure. Inhibition of tubular reabsorption is well documented but the underlying mechanisms are incompletely defined.[40] Intrarenal paracrine signaling by ATP may mediate some aspects of the pressure-natriuresis

response[25], but the current study suggests that tonic P2X7R activation suppresses this relationship and is detrimental to blood pressure control in ANGII-treated rats. P2X7R antagonism caused a marked improvement in the pressure-natriuresis. The leftward shift in the relationship can be attributed to vasodilation; several mechanisms could account for the improved gradient of the response. First, interstitial inflammation suppresses pressure natriuresis[48] and the beneficial effect of BBG could reflect acute anti-inflammatory actions of P2X7R inhibition. This seems unlikely to be the major effect here because the response was too rapid and inflammation in our model is mild. Second, P2X7R activation increases the generation of reactive oxygen species[49], which reduce blood flow in the medullary *vasa recta* and impair the pressure-natriuresis response.[50] Third, P2X7R blockade increases blood flow directly by alleviating constriction of the *vasa recta*[26] This is an attractive hypothesis, because extracellular ATP is an established regulator of microvascular function.[51] P2X7R is expressed in the *vasa recta*[25] and causes constriction of the abluminal pericytes that control blood flow through the capillary network.[26] Our data offer some support for this hypothesis because systemic intravenous injection of AZ11657312 increased medullary microvascular perfusion. Nevertheless, this effect was only observed under high ANGII tone, a condition that increases interstitial ATP concentrations,[28] and it may be that the influence of P2X7R on microvasculature function is normally small. P2X7R antagonism also improved renal oxygenation in ANGII-treated rats and this beneficial effect was particularly evident in high R2* cluster, *i.e.* in areas of the kidney that are relatively poorly oxygenated. It is not, however, possible, to attribute this improved oxygenation solely

to increased tissue perfusion as a reduction in active tubular transport may also be a contributory factor.

Recent studies have reported a beneficial effect of P2X7R antagonism in the Dahl salt-sensitive rat model of hypertension[35] and of P2X7R ablation in the DOCA model in mice.[22] BBG almost abolished interstitial fibrosis and albuminuria in these settings and this cannot be entirely attributed to a reduction in barotrauma, because the reduction in blood pressure was modest. Interestingly, there was also a reduction in the number of infiltrating immune cells (mainly macrophages) in the rats treated with BBG, and the authors postulated that this was the mechanism of renoprotection. The generation of a hypoxic microenvironment in the renal medulla is pro-inflammatory and may be an early pathway to kidney injury.[52]

Taken together, these studies suggest that P2X7R antagonism may affect both inflammation and vasoconstriction. First, a reduction in blood pressure ameliorates barotrauma of the glomerulus and renal microvasculature; second, increased perfusion of the renal medulla restores blood pressure control and minimizes hypoxia; third, P2X7R antagonism may also reduce the immune cell response and local inflammation. Thus, P2X7R antagonism may have broad therapeutic value in renal disease.

METHODS

Procedures were performed under the 1986 UK Home Office Animals (Scientific Procedures) Act, following local ethical review. Experiments were performed on male F344/NCrl rats (Charles River, UK) with free access to standard chow (0.25% sodium) and water. Osmotic minipumps (model 2002; Alzet, UK) were implanted subcutaneously under isofluorane anesthetic with vetergesic analgesic. Minipumps delivered ANGII (30ng/min) or saline vehicle and experiments were performed 11-14 days after implant.

Acute pressure-natriuresis was measured under terminal thiobutabarbital anesthesia (Inactin; 120mg/kg IP). The right jugular vein was canulated for infusion of 0.9% NaCl (50µl/min/100g during surgery; 33µl/min/100g after surgery) or saline containing Brilliant Blue G (50µg/min/100g). The left femoral artery was cannulated to record blood pressure (MLT844; Capto, Norway) at 1 kHz (Powerlab; AD Instruments, UK). A midline laparotomy was performed and a Doppler transit time probe (MA1PRB; Transonic, USA) was placed around the around the left renal artery and covered in acoustic gel to optimize coupling. Core body temperature was maintained at 37°C using a servo-controlled heating blanket. Surgical preparation took ~1.5 hours and was followed by a 60-minute equilibration period. Urine collections were then made over 3 sequential periods, each of 30 minutes. After the first (baseline) collection, blood pressure was acutely increased by ligation of the superior mesenteric artery and urine collected. The coeliac artery was then ligated, after which the final urine collection was made. For each period urine was collected under mineral oil via a bladder catheter to determine flow rate. Urinary sodium concentration was measured by ion-sensitive electrode (InSight; QBC Europe). In a

subset of rats (n=4 per group), creatinine concentration was measured in each urine sample and in a plasma sample drawn at the end of the experiment. Creatinine was determined using the creatininase/creatinase specific enzymatic method using a commercial kit (Alpha Laboratories Ltd, UK) adapted for use on a Cobas Fara centrifugal analyser (Roche Diagnostics Ltd, UK). Creatinine clearance was calculated to provide an estimate of glomerular filtration rate.

AZ11657312 cumulative dose-response studies were performed in anaesthetized rats as above. The renal jugular vein was cannulated (PE50 tubing) for infusion of 0.9% NaCl and a second cannula (PE10) inserted for drug injection. Blood pressure was measured at the left femoral artery, renal blood flow at the left renal artery, as before. Additionally, an optical Doppler flux probe (Type M monfibre probe, Transonic, USA) was advanced into the renal medulla to measure microvascular perfusion. Probe position was confirmed post-mortem. Surgical preparation took ~1.5 hours and was followed by a 60-minute equilibration period. AZ11657312 was injected intravenously in a 0.1ml of vehicle (1% DMSO in saline) and doses were administered in ascending order, preceded and followed by injection of the vehicle alone. Measurements were made over a period of 30-60 minutes.

BOLD MRI was performed as previously described.[34] Three sequential baseline scans were performed, after which BBG (40mg/kg) was administered by tail vein injection. Ten further scans were obtained. Total scan time was ~40mins. T2* maps were constructed followed by three-step image registration (global & intensity based) and motion artifact (Hampel identifier) quality control.

k-means clustering analysis. Automated image segmentation was performed using a *k*-means clustering algorithm as previously described.[34] This identifies *k*-clusters

within a multi-dimensional space using Euclidean distance. Given a set of points, the target of the algorithm is to find k cluster-centers such that the sum of square distances of each point to its closest cluster centre is a minimum. The (local) minimum is searched for in an iterative manner, the two steps of which are *1*) the association of the points with their closest cluster centers, and *2*) the updating of the cluster centers such that the sum of square distances to the associated points is minimized. Since the final cluster configuration can be dependent on that of the initial cluster configuration, we previously established the lowest number insensitive to the starting conditions. The present dataset found 10 random initial conditions to fit this condition, thus for each scan set we ran the algorithm and saved the final configuration as that with the lowest sum of intracluster distances.

Our previous analysis was performed using k=1, 2, 3 up to 13 in order to identify the value of k such that the increase in explained variance of k+1 clusters was <50% of the additional variance explained by the kth cluster. k =2 was chosen, as the addition of a third cluster did not contribute sufficiently to an increase in explained variance. Each time series required ~50ms to converge when run in MATLAB on a standard desktop computer.

Renal injury scoring was performed on hematoxylin & eosin stained sections: all embedded tissues were sectioned and stained in the same run. Vacuoles were close to circular edges and displaced nuclei. Total number of vacuoles counted per vessel was normalised to vessel thickness: this scoring was performed under single-blind conditions.

Macrophage staining was performed using CD68 antibody (Serotec, UK; 1:500) and quantified using the automated detection tool ImmunoRatio normalised to

haematoxylin positive staining within individual glomeruli. At least 30 glomeruli were analysed per experimental group. Immunolocalisation of P2X7R used a rabbit anti-rat polyclonal antibody (APR-004, Alomone Laboratory, Israel) at 1:2000. This antibody is raised against an epitope on the intracellular c-terminal of the receptor, a region unique among P2X receptors and has been validated in both heterologous expression systems in in P2X7R null mice (see[53] for review).

Statistics: Data are presented as mean ± SEM or median and inter-quartile range, as stated. Statistical analysis was by repeated measures ANOVA, t-test or Mann Whitney U test, as stated. For ANOVA, post hoc comparisons were made using Sidak's multiple comparison test.

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DISCLOSURE

RJU is currently on secondment as Chief Scientist to Cardiovascular and Metabolic Diseases (iMed CVMD) R&D, AstraZeneca, Mölndal, Sweden. FWKT has received research funding from AstraZeneca.

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Figure 1: Renal function is improved by P2X7R antagonism. The selective P2X7R antagonist Brilliant Blue G (BBG**)** was infused intravenously in rats chronically infused with ANG II. BBG infusion improved A) mean arterial blood pressure; B) urinary sodium excretion; C) the acute pressure natriuresis response and; D) the pressure diuresis relationship in ANG II-treated rats. In A) and B) data are mean ±SEM; in C and D, individual points are plotted with the linear regression line and 95% confidence interval. Experiments were performed in control rats (n=7; open circles), ANGII-treated rats (n=5; closed squares) and ANGII+BBG (n=5, grey squares).

Figure 2: P2X7R localization in renal vasculature. P2X7R was localized to the endothelium of preglomerular arteries. A Segmental artery from A) Vehicle and B) ANGII-treated rat is shown. Focal expression of P2X7R (indicated by white arrows) in the vascular smooth muscle was observed in ANGII treated rats, show in Figure 2Bi. P2X7R expression was also observed in interlobar arteries (C= vehicle; D= ANGII) and afferent arterioles (E=vehicle; F= ANGII). Scale bars indicate 20μm.

Figure 3: **Chronic ANGII infusion induces renal injury**. ANGII promoted vacuolization of myocytes in pre-glomerular arteries, particularly the larger segmental and interlobar arteries. A and B show exemplar images from ANGII-treated rats; C is from a vehicle treated rat. This injury was scored in a single blind fashion and was significantly higher in ANGII treated rats (Panel D; Data are mean ±SEM and comparison was made using unpaired T-test). Chronic ANGII infusion caused a modest increase in the number of glomeruli containing ED-1 positive macrophage staining (Panel E. Individual data are shown from control and ANGII-treated rats

along with the median and inter-quartile range; comparisons were made using Mann-Whitney test). P values as shown are two-tailed.

Figure 4: P2X7R antagonism increases renal perfusion. The specific P2X7R antagonist AZ11657312 caused a dose-dependent A) reduction in mean arterial blood pressure; B) increase in renal artery blood flow and C) increase in perfusion of the renal medulla in ANGII-treated rats. The area under the curve for each dose of drug was calculated and normalized to the effect of the saline vehicle in each rat. Data are mean ±SEM. ANOVA indicated a significant effect of AZ11657312 ($P<0.001$) on each variable and Sidak's multiple comparison test was used for post-hoc comparisons. *$P<0.05$ for antagonist versus vehicle in the control group (n=5). $^{\dagger}P<0.05$, $^{\dagger\dagger}P<0.01$, $^{\dagger\dagger\dagger}P<0.001$ and $^{\dagger\dagger\dagger\dagger}P<0.0001$ for antagonist versus vehicle in the ANGII group (n=6).

Figure 5: Renal vascular effects of P2X7R antagonism are nitric oxide dependent. The effect of the specific P2X7R antagonist AZ11657312 was assessed in ANGII-treated rats (n=6) before and after inhibition of nitric oxide synthase. A) mean arterial blood pressure; (B), renal blood flow and (C) medullary tissue perfusion (C). The area under the curve for each dose of drug was calculated and normalized to the effect of the saline vehicle in each rat. Data are mean ±SEM. Data are mean ±SEM. ANOVA indicated a significant effect of AZ11657312 ($P<0.001$) on each variable and Sidak's multiple comparison test was used for post-hoc comparisons. *$P<0.05$, **$P<0.01$ and ****$P<0.0001$ for antagonist dose compared with vehicle in the ANGII group. $^{\dagger}P<0.05$, $^{\dagger\dagger}P<0.01$, $^{\dagger\dagger\dagger}P<0.001$ and $^{\dagger\dagger\dagger\dagger}P<0.0001$ compared with vehicle after infusion of L-NAME to inhibit nitric oxide production.

Figure 6: P2X7R antagonism improves renal oxygenation in ANGII-treated rats. Intravenous injection of the selective P2X7R antagonist Brilliant Blue G (shown by arrow) reduced R2* (i.e. improved pO_2) in ANGII-treated rats (black squares; n=6) but not in control rats (open circles; n=6). The effect was more pronounced in A) areas of low basal oxygenation compared with B) the cluster of high oxygenation areas. For each rat, the change in R2* induced by Brilliant Blue G is expressed as a percentage of the baseline recordings. Data are mean±SEM. ANOVA indicated a significant effect of the P2X7R antagonist ($P<0.05$) and Sidak's multiple comparison test was used for post-hoc comparison. $^{\dagger}P<0.05$, $^{\dagger\dagger}P<0.01$, $^{\dagger\dagger\dagger}P<0.001$ compared with baseline in the ANGII group.