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Purinergic receptors contribute to early mesangial cell transformation and renal vessel hypertrophy during angiotensin II-induced hypertension

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Abstract

Chronic ANG II infusions lead to increases in intrarenal ANG II levels, hypertension, and tissue injury. Increased blood pressure also elicits increases in renal interstitial fluid (RIF) ATP concentrations that stimulate cell proliferation. We evaluated the contribution of purinergic receptor activation to ANG II-induced renal injury in rats by treating with clopidogrel, a P2Y₁₂ receptor blocker, or with PPADS, a nonselective P2 receptor blocker. α -Actin expression in mesangial cells, afferent arteriolar wall thickness (AAWT), cortical cell proliferation, and macrophage infiltration were used as early markers of renal injury. Clopidogrel and PPADS did not alter blood pressure, renin or kidney ANG II content. α -Actin expression increased from control of $0.6 \pm 0.4\%$ of mesangial area to $6.3 \pm 1.9\%$ in ANG II-infused rats and this response was prevented by clopidogrel ($0.4 \pm 0.2\%$) and PPADS. The increase in AAWT from 4.7 ± 0.1 to 6.0 ± 0.1 mm in ANG II rats was also prevented by clopidogrel (4.8 ± 0.1 mm) and PPADS. ANG II infusion led to interstitial macrophage infiltration (105 ± 16 vs. 62 ± 4 cell/mm²) and tubular proliferation (71 ± 15 vs. 20 ± 4 cell/mm²) and these effects were prevented by clopidogrel (52 ± 4 and 36 ± 3 cell/mm²) and PPADS. RIF ATP levels were higher in ANG II-infused rats than in control rats (11.8 ± 1.9 vs. 5.6 ± 0.6 nmol/l, $P < 0.05$). The results suggest that activation of vascular and glomerular purinergic P2 receptors may contribute to the mesangial cell transformation, renal inflammation, and vascular hypertrophy observed in ANG II-dependent hypertension.

Keywords

extracellular ATP; renal inflammation; vascular hypertrophy

Ang II-Dependent Hypertension is characterized by increases in intrarenal ANG II content and renal functional impairment with variable degrees of injury to several organs and tissues, including heart, kidney, and blood vessels (7,16,23,25,³¹,38,42,47,50). The ANG II-associated tissue injury occurs more consistently in a setting of elevated arterial pressure suggesting that factors existing in hypertensive, but not normotensive, conditions may be contributing to the damage observed during hypertension caused by chronic ANG II infusions. While mechanical forces caused by the elevated arterial pressures directly cause baromechanical trauma (1), elevated arterial pressure may independently activate paracrine systems that contribute to the ANG II-mediated vascular and tissue injury (38,47). Various proliferative agents are capable of inducing vascular hypertrophy, mesangial activation, or cortical renal injury (38). These independent factors, directly stimulated by the elevated arterial pressure, may synergize with the elevated intrarenal ANG II levels to elicit renal injury.

Extracellular ATP is now recognized as a powerful paracrine agent that exerts acute and long-term actions via activation of one or more members of the purinergic P2 receptor family (13–15,26,43). Stimulated endothelial cells release endogenous nucleotides, including ATP (48). In addition, renal interstitial fluid (RIF) ATP concentrations increase in response to increases in renal arterial pressure and regulate renal vascular resistance (14,27–29). With sustained increases, extracellular ATP is a proliferative agent, capable of inducing vascular smooth muscle hypertrophy and hyperplasia (6,12,40,43). In the kidney, these actions are mediated by the purinergic receptors, P2X and P2Y, which are present in the renal vasculature (3,41).

There is abundant evidence that ANG II-induced activation of inflammatory mediators and immune cells contribute importantly to the pathogenesis of renal tissue injury in various hypertensive states (4,8,11,33,36). Particularly, there is growing awareness that hypertension in general and hypertension combined with increased ANG II levels in particular are associated with macrophage infiltration and proliferative activity in renal tissue (4,8,11,31,36). Accordingly, immunosuppressive drugs are able to reduce blood pressure in experimental hypertension (10). Therefore, we investigated the presence of cellular and inflammatory mechanisms as surrogate markers of renal damage occurring during induction of hypertension.

In this paper, we addressed the hypothesis that increased renal interstitial ATP levels contribute to proliferation of vascular smooth muscle cells and promote renal injury in ANG II-dependent hypertension. For the present study, we focused on early phenomena that might allow the identification of key initiating events that ultimately lead to more generalized renal pathology. An early sign of renal injury in hypertension is increased α -actin expression in glomerular mesangial cells predisposing to matrix deposition and glomerulosclerosis (19,24). Accordingly, we tested the ability of pyridoxal-phosphate-6-azophenyl-z',4'-disulphonic acid tetrasodium (PPADS), a nonspecific P2 receptor blocker, and of clopidogrel, a P2Y₁₂ purinergic blocker, to ameliorate mesangial transformation induced by ANG II (39,46,49). Because ATP may act through many different types of receptors and because purinergic blockade using the nonspecific P2 receptor blocker PPADS has been shown to inhibit mesangial cell proliferation in a model of glomerulonephritis (37), we tested the ability of PPADS to prevent the early renal injury commonly associated with ANG II-dependent hypertension. Clopidogrel is a specific blocker of P2Y₁₂ receptors (9,39) that is used in clinical practice to inhibit platelet aggregation. Accordingly, we tested the ability of Clopidogrel, as well as PPADS, to ameliorate mesangial cell transformation, preglomerular vessel hypertrophy, and cortical inflammation and cell proliferation, early phenomena occurring in several different models of hypertension (2,7,34).

Materials and Methods

All experimental procedures were approved by Tulane University's Institutional Animal Care and Use Committee. Three separate protocols were used in this study. ANG II was infused chronically using osmotic minipumps (Alzet, Alza, Cupertino, CA) implanted in rats anesthetized with sodium isoflurane, 5% in oxygen vol/vol (IsoFlo, Abbott, IL).

Protocol I

Fifteen male Sprague-Dawley rats (Charles River, Wilmington, MA) weighing 200–250 g were divided into three groups ($n = 5/\text{group}$): control, consisting of sham-operated animals; ANG II, consisting of rats receiving ANG II (80 ng/min) via osmotic minipump for 14 days; and ANG II + clopidogrel (CLOP), consisting of animals receiving both ANG II (80 ng/min) and CLOP (20 mg $\cdot$ kg⁻¹ $\cdot$ day⁻¹ as clopidogrel tablets dissolved in drinking water, Plavix, Bristol-Myers Squibb/Sanofi Pharmaceutical, Bridgewater, NJ) for 14 days. This dose is known to be effective (5) and safe based on results from experiments testing drug toxicity in rats (35).

Protocol II

Fifteen male Sprague-Dawley rats were divided into three groups ($n = 5/\text{group}$): control ($n = 5$), consisting of sham-operated animals; ANG II, consisting of rats receiving ANG II (80 ng/min) through a subcutaneously implanted osmotic minipump for 14 days; and ANG II + PPADS, consisting of animals receiving ANG II (80 ng/min) and PPADS (20 mg/day ip; Sigma, St. Louis, MO). The employment of PPADS in vivo is based on studies in a model of glomerulonephritis (37).

Protocol III

To determine whether chronic infusion of ANG II leads to increased RIF concentrations of ATP, 16 male Sprague-Dawley rats were divided into two groups to measure RIF ATP: control ($n = 8$), consisting of sham-operated animals; and ANG II ($n = 8$), consisting of rats receiving ANG II (80 ng/min) via osmotic minipump for 14 days.

In *protocols I and II*, the rats were followed for 14 days and a 24-h urine specimen was collected on *day 13* to determine albumin excretion. Systolic blood pressure (SBP) was measured by tail-cuff plethysmography (Visitech, BP2000, Visitech Systems, Apex, NC) for 3 consecutive days before surgery and death. On *day 14*, the rats were killed by conscious decapitation with collection of trunk blood into chilled tubes containing EDTA (5 mmol/l), enalaprilat (20 μ mol/l), pepstatin (20 μ mol/l), PMSF (10 μ mol/l), and 1,10-phenanthroline (1.25 mmol/l, all from Sigma) and stored at -20°C until assayed using RIA for plasma renin activity (PRA) and ANG II (Phoenix Pharmaceuticals, Burlingame, CA). Kidneys were removed, divided, and fixed in buffered formalin for histological analysis, in RNA-later (Ambion) for RT-PCR, or homogenized in cold methanol and processed for measurements of renal ANG II as previously described (21,51). Urinary albumin concentration was measured using a commercially available ELISA kit for rat albumin (Cayman Chemical, Ann Arbor, MI).

Immunohistochemical analysis of α -smooth muscle actin

The immunohistochemical studies were performed using 4- μ m-thick, paraffin-embedded renal sections. Sections were mounted on glass slides, deparaffinized, and rehydrated using standard techniques. Sections were then exposed to microwave irradiation in 0.01 M citrate buffer to enhance antigen retrieval and preincubated with 5% normal horse serum in Tris-buffered saline to prevent nonspecific protein binding and with avidin and biotin solutions to block nonspecific binding of these compounds (Blocking Kit, Vector Labs, Burlingame,

CA). Incubation with the primary antibody, a monoclonal mouse antibody (Sigma), was carried out overnight at 4°C in a humidified chamber. Detection was done by an indirect avidin-biotin peroxidase technique. After being washed, the sections were incubated at room temperature with rat-adsorbed biotinylated anti-mouse (Vector Labs) for 45 min, and then with avidin-biotin-horseradish peroxidase (Vector Labs) for an additional 30 min. Sections were incubated with a freshly prepared substrate, consisting of DAB and hydrogen peroxide and developed in a dark chamber protected from light. Negative control experiments were performed by omitting incubation with the primary antibody. Quantification of glomerular (mesangial) α -actin expression was performed using an image analysis software (Image-Pro Plus, MediaCybernetics, Bethesda, MD) after image acquisition using a digital system (Magnafire, GTI, Tempe, AZ) coupled to an optical microscope (Olympus BX-50, Olympus Optical, Tokyo, Japan). Glomerular actin expression is described as percentage of stained area relative to total glomerular area. In each section, at least 30 glomeruli were analyzed. The thickness of the wall of afferent arterioles was measured as previously described (31).

Immunohistochemical analysis of proliferating cell nuclear antigen and ED-1 (macrophages)

To evaluate the participation of inflammatory process in the development of renal damage in ANG II-induced hypertension as well as the effect of purinergic blockade on this process, immunohistochemistry experiments for macrophage infiltration and cell proliferation were performed.

To detect proliferating cells, renal sections were analyzed using a monoclonal mouse antibody (Dako) specific for the proliferating cell nuclear antigen (PCNA) and an indirect streptavidin-biotin alkaline phosphatase technique. The technique is similar to the peroxidase method described above but employs the streptavidin-biotin-alkaline phosphatase complex (Dako) instead of avidin-biotin horseradish peroxidase (HRP). Moreover, in place of DAB sections were incubated with a freshly prepared substrate, consisting of naphthol AS-MX-Phosphate (Sigma) and fast red dye (Sigma), counterstained with Mayer's hemalaum (Merck, Darmstadt, Germany) and covered with glycergel (Dako).

To identify macrophages in the kidney tissue, a monoclonal mouse anti-rat ED-1 antibody (Serotec, Oxford, UK) was employed, using an avidin-biotinylated HRP method as mentioned above.

The quantitation of cortical interstitial and tubular PCNA-positive cells was carried out in a blind fashion under $\times 400$ magnification and expressed as cells per millimeter squared. For each section, 30 microscopic fields, each corresponding to an area of 0.13 mm², were examined. The number of cells located at the glomeruli and tubules was also determined and expressed as cells per glomerulus. The quantitation of cortical interstitial and tubular ED-1-positive cells in the interstitium was expressed as cells per millimeter squared. Thirty microscopic fields, each corresponding to an area of 0.13 mm², were examined. The number of cells located at the glomerulus was also determined and expressed as cells per glomerulus.

RT-PCR for P2Y₁₂ receptors

Half of one kidney from each rat was placed into RNAlater (Ambion, Austin, TX) and kept at -80°C. Total RNA was extracted using a commercially available kit (Qiagen, Valencia, CA) and treated with DNase I (Invitrogen, Carlsbad, CA) to remove contaminating genomic DNA. Real-time RT-PCR was performed as previously described (22,32,46). Quantitative real-time RT-PCR was conducted in the Mx3000P equipment (Stratagene, La Jolla, CA) using MxPro QPCR software (Stratagene) and Brilliant 1-Step QRT-PCR master

mix kit as reagent (Stratagene). Dual label was 5': 5-HEX, 3': BHQ-2 for control gene and 5': 6-FAM, 3': BHQ-1 for target genes. The program conditions were *step 1*: 50°C, 30 min, 1 cycle in the first step; 95°C, 10 min, 1 cycle in *step 2*; and 95°C, 15 s followed by 60°C, 1 min, 40 cycles in *step 3*. The base pair sequence of the primers and the probes for real-time RT-PCR for GAPDH were CAGAACATCATCCCTGCATC, CTGCTTCACCACCTTCTTGA, CCTGGAGAAACC-TGCCAAGTATGATGA, representing sense primer, antisense primer, and probe, respectively. Similarly, the sequences for P2Y₁₂ were GATTCTTTCTGTTGCCATCT, CCGACTTCAAGAAAGAACAT, and CCTGCTGTCACTGCCTAACATGAT, representing sense primer, antisense primer, and probe, respectively. The quantity of P2Y₁₂ mRNA expression was normalized by that of GAPDH mRNA expression.

RIF ATP concentration

To determine whether chronic infusion of ANG II leads to increased RIF concentrations of ATP, control and ANG II-infused rats were anesthetized with pentobarbital sodium, the left kidney was exposed, and microdialysis probes were inserted into the renal cortex to collect RIF for ATP measurements as previously described (27). At 14 days of ANG II infusion or sham operation, the rats were anesthetized using pentobarbital sodium (50 mg/kg ip), a tracheotomy was performed, and a 1% albumin in saline solution at a rate of 20 µl/min was infused to compensate for surgically induced fluid losses. Arterial pressure was measured via a catheter inserted into one carotid artery. After a flank incision, the left kidney was exposed and microdialysis probes were inserted and perfused with Ringer solution (pH 7.4) at a rate of 3 µl/min. After an equilibration period of 60 min, 10-min collections of RIF were obtained. The dialysate samples were directly collected from outflow steel tubing of two microdialysis probes and were stored at -70°C before analysis. ATP concentration was measured using a commercially available kit employing luciferin-luciferase luminescence technique (Sigma).

Statistical analysis

One-way ANOVA with unpaired comparisons according to the Newman-Keuls formulation was used in this study. As the results for PRA and plasma and kidney ANG II were repeatedly similar, the rats from *protocols I* and *II* were regrouped in controls, ANG II infusion, and ANG II with treatment. For comparisons between two groups in *protocol IV*, Student's *t*-test with Welch's correction for unequal variances was employed. Correlation using Pearson formulation was utilized to evaluate the correlation between RIF ATP and mean arterial pressure (MAP). *P* values <0.05 were considered significant.

Results

SBP

Average SBP remained normal in control rats (123 ± 4 mmHg) but increased to 193 ± 7 mmHg by *day 14* of ANG II infusion ($P < 0.01$ vs. control; Table 1). SBP was also elevated to the same extent in the ANG II + CLOP group (193 ± 4 mmHg). In *protocol II*, SBP was 117 ± 3 mmHg in control rats and ANG II infusion increased blood pressure to 199 ± 6 mmHg. PPADS administration to ANG II-infused rats did not alter the ANG II-induced hypertension 195 ± 7 mmHg ($P < 0.05$ for ANG II and PPADS vs. control). SBP in control animals did not differ from their baseline data, measured 1 day before surgery (Table 1). In *protocol III*, MAP in the anesthetized rats averaged 111 ± 3 mmHg in controls and 144 ± 4 mmHg in animals receiving chronic ANG II infusion for 14 days ($P < 0.001$).

Urinary albumin excretion

As shown in Table 1, urinary albumin was increased after 13 days of ANG II infusion, reaching 9.67 ± 2.03 vs. 0.13 ± 0.02 mg/day in controls. CLOP treatment to ANG II-infused rats failed to prevent the increase in albumin excretion (7.45 ± 1.16 mg/day). In *protocol II*, urinary albumin was increased by ANG II infusion (7.9 ± 2.3 vs. 0.5 ± 0.1 mg/day in controls). PPADS treatment to ANG II-infused rats slightly diminished but did not prevent the increased albumin excretion (5.0 ± 1.6).

PRA and ANG II measurements

As mentioned above, the results for PRA and plasma and kidney ANG II were not different between groups; therefore, the rats from *protocols I* and *II* were regrouped and analyzed as controls, ANG II infusion, and ANG II with treatment. As expected, PRA was suppressed by ANG II infusion when compared with control rats (0.04 ± 0.02 vs. 5.55 ± 0.45 ng $\cdot$ ml⁻¹ $\cdot$ h⁻¹, $P < 0.05$). Treatment with anti-purinergics did not alter the PRA response to ANG II infusion (0.06 ± 0.01 ng $\cdot$ ml⁻¹ $\cdot$ h⁻¹) and there was no difference between the CLOP and PPADS treatments.

Plasma ANG II concentrations did not differ among the different groups in control animals and in rats receiving ANG II infusion alone or in combination with CLOP or PPADS administration. Accordingly, plasma ANG II levels were 96 ± 27 fmol/ml in controls, 61 ± 13 fmol/ml in ANG II infusion, and 95 ± 5 fmol/ml in ANG II infusion + treatment. The differences were not statistically significant. However, kidney ANG II content was significantly increased in ANG II-infused rats and in rats receiving ANG II infusion plus purinergic receptor blockers compared with controls. Indeed, kidney ANG II content was increased by ANG II infusion when compared with controls (327 ± 46 vs. 133 ± 19 fmol/g, $P < 0.05$) and rats receiving both ANG II and CLOP had a similar response (435 ± 36 fmol/g, $P < 0.05$ vs. control).

Mesangial α -smooth muscle actin expression

Representative images are shown in Fig. 1 and the quantitative results are presented in Fig. 2, A and B. Mesangial α -actin expression increased from $0.6 \pm 0.4\%$ of glomerular area in the control sham group to $6.3 \pm 1.9\%$ in the ANG II group ($P < 0.05$). CLOP prevented the increased α -actin expression ($0.4 \pm 0.2\%$, $P < 0.05$). In *protocol II*, α -actin expression in the mesangium represented $0.4 \pm 0.2\%$ of mesangial area in the controls; as shown in Fig. 2B, infusion of ANG II augmented this value to $3.6 \pm 0.8\%$ ($P < 0.01$ vs. control), and treatment with PPADS prevented this increase ($0.9 \pm 0.3\%$, $P < 0.01$ vs. ANG II).

Afferent arteriole wall thickness

As shown in Fig. 3A, afferent arteriole wall thickness averaged 4.7 ± 0.1 μ m in control rats and was significantly greater (6.0 ± 0.1 μ m) in the ANG II group ($P < 0.05$ vs. control). CLOP treatment prevented the ANG II-induced increases in afferent arteriolar wall thickness (4.8 ± 0.1 μ m; $P < 0.05$ vs. ANG II; Fig. 1). Similarly, in *protocol II*, afferent arteriole wall thickness was greater in rats receiving ANG II (6.3 ± 0.2 μ m) compared with controls (4.8 ± 0.1 μ m; $P < 0.01$ vs. control). Rats receiving PPADS did not exhibit a significant increase in arteriole thickness averaging 5.1 ± 0.3 μ m ($P < 0.01$ vs. ANG II; Fig. 3B).

Renal cell proliferation

Analysis of renal PCNA expression revealed that cell proliferation was higher in cortical tubules of ANG II-infused rats compared with normotensive rats (71 ± 15 vs. 20 ± 4 cell/mm²). As shown in Fig. 6A (see Fig. 6) and qualitatively in Figs. 4, A–C, tubular

proliferation was particularly noticeable in focal areas of tubular dilation. CLOP diminished this expression (36 ± 3 cell/mm²). The number of proliferating cells in the cortical interstitium of ANG II-infused rats was increased compared with controls (50 ± 13 vs. 15 ± 4 cell/mm²), and CLOP reduced the interstitial cell proliferation (17 ± 5 cell/mm²; Fig. 6B and representative slides in Fig. 4, A, B, and C). Rats receiving ANG II infusion had more glomerular cell proliferation than controls (2.4 ± 0.2 vs. 1.0 ± 0.2 cell/glom.) and CLOP diminished cellular proliferation in the glomeruli (1.7 ± 0.2 cell/glom.). The corresponding results are shown in Fig. 6C, and images illustrating glomerular proliferation are shown in Figs. 4, D, E, and F. Similarly, PPADS reduced the tubular, interstitial, and glomerular cell proliferation induced by ANG II infusion (Table 2).

Renal macrophage infiltration

Figure 7A depicts the immunohistochemical analysis of the renal cortical tissue for the ED-1 and PCNA antigens at 14 days of ANG II infusion. The density of cells expressing the macrophage-specific ED-1 antigen was low in the renal cortical interstitium of sham rats (62 ± 4 cell/mm²) and increased significantly in the interstitium of ANG II-infused rats (105 ± 16 cell/mm²). CLOP prevented the increased interstitial ED-1 expression in treated rats (52 ± 4 cell/mm²; Fig. 7A and representative slides in Fig. 5, A, B, and C). Similarly, PPADS also diminished the interstitial macrophage accumulation induced by ANG II infusion (Table 2). It should be noted, however, that ANG II infusion did not increase macrophage infiltration of glomeruli, and neither CLOP nor PPADS altered this pattern (Fig. 7B and Table 2, respectively).

Renal P2Y₁₂ receptor mRNA expression

P2Y₁₂ receptor mRNA expression measured by RT-PCR demonstrated the presence of P2Y₁₂ receptors in kidney tissue. Sham-operated animals showed a P2Y₁₂/GAPDH ratio equal to 0.75 ± 0.12 , compared with 0.55 ± 0.14 in rats receiving ANG II infusion and 0.50 ± 0.10 in rats receiving ANG II and treated with CLOP. Neither ANG II infusion nor treatment with CLOP altered the P2Y₁₂/GAPDH ratio.

RIF ATP concentration

Rats infused chronically with ANG II for 14 days showed higher RIF ATP concentrations than control rats (Fig. 8A). RIF ATP was 5.6 ± 0.6 nmol/l in control rats and 11.8 ± 1.9 nmol/l in ANG II-infused rats with hypertension ($P < 0.05$). RIF ATP, ranging from 3.6 to 19.0 nmol/l, correlated with MAP, ranging from 96 to 160 mmHg, with a correlation coefficient $R^2 = 0.45$ ($P < 0.01$; Fig. 8B).

Discussion

ANG II-dependent hypertension is characterized by elevated intrarenal and RIF ANG II levels and a proliferative response leading to renal injury (20,21,30,31,50). The general finding that elevated intrarenal ANG II levels alone in the absence of elevated arterial pressure are not associated with perceptible renal injury and that ANG II-induced injury usually occurs in a setting of elevated arterial pressure (16,23,38,45,47,50) suggests that elevated intrarenal ANG II alone is not sufficient to cause renal injury. Thus additional factors such as growth factors, cytokines, and other paracrine agents augmented by the biomechanical stimulus caused by increased arterial pressure may contribute to the proliferative response. Because increased renal arterial pressure elicits increases in RIF ATP concentrations (13,14,26–28), pressure-induced increases in RIF ATP may contribute to the long-term proliferative responses leading to renal injury and fibrosis. Several studies demonstrated that ATP has growth stimulating and proliferative effects on various tissues (6,40,43). Accordingly, we developed the hypothesis that the increased blood pressure

caused by the elevated intrarenal ANG II levels elicits chronic elevations in RIF ATP which contribute to the proliferative response associated with ANG II-dependent hypertension. In accord with this hypothesis, we found that chronically hypertensive rats had higher cortical interstitial ATP than control rats and that MAP correlated with RIF ATP.

It is important to note that P2Y purinergic receptor expression has been shown to be increased or expressed de novo in hypertension (44,46). The presence of P2Y₁₂ receptors in renal tissue provides the molecular basis to substantiate the role of purines in the early renal damage occurring during ANG II-dependent hypertension. This role is strongly corroborated by the ability of the purinergic receptor blockers, CLOP and PPADS, to reduce or prevent mesangial cell transformation, afferent arteriole thickening, renal cortical proliferation, and macrophage infiltration induced by chronic ANG II infusions. These results support the contention that purinergic receptor activation contributes significantly to the pathologic phenomena occurring in ANG II-dependent hypertension. While P2Y₁₂ receptors are thought to be activated primarily by ADP, they may also be activated by ATP (46). Alternatively, ATP may be converted in the interstitial space to ADP, which in turn activates the P2Y₁₂ receptor. Regardless of the ligand responsible for initiating the activation of P2Y₁₂, the present study clearly demonstrates that blockade of the P2Y receptor with CLOP during ANG II-dependent hypertension alleviates the corresponding injury suggesting that activation of purinergic receptors is an important contributing factor to tissue injuries induced by ANG II.

Although the purinergic receptor, P2Y₁₂, is expressed primarily in platelets and brain, findings by Wang et al. (44) and the present study indicate that P2Y₁₂ receptors are expressed in renal and vascular tissue. However, we cannot exclude the possibility that resident platelets within interstitial and glomerular areas also mediate renal damage after activation of platelet P2Y₁₂ receptors. The previously reported finding that platelet-depleted rats are protected from renal damage in immune complex nephritis is consistent with this possibility (17). However, regardless of the intervening events, the current study demonstrates that purinergic signaling may have an important role in mediating hypertensive-related renal injury. Because treatment with CLOP or PPADS did not prevent or reduce the ANG II-mediated increases in arterial pressure, the present study also indicates that these initial events are mediated by paracrine factors rather than the direct baromechanical actions of the elevated arterial pressure. Zoja et al. (49) showed that Ticlopidine, another blocker of P2Y₁₂, diminished renal injury in the renal ablation model. Interestingly, purinergic receptor antagonism did not alleviate the albuminuria, which is commonly correlated with hypertension-induced renal injury and progressive renal functional impairment. Presumably, mesangial cell transformation does not necessarily correlate with epithelial podocyte function or other structures involved in glomerular protein restriction. It is likely that the maintained arterial and glomerular pressures provide the functional basis for the albuminuria.

It should be pointed out that the events evaluated in this study occur very early in the development of ANG II-induced hypertension. Transformation of cells of fibroblast lineage is a key phenomenon in renal damage, as the modified cells acquire synthetic phenotype, enabling them to deposit matrix in the interstitium. Moreover, the acquisition of contractile protein allows those cells to shrink the surrounding region forming scar tissue. Preglomerular vessels might well be a site where high ANG II and RIF ATP interact to cause vessel hypertrophy. This model mirrors human essential hypertension in which vascular reactivity evolves into arteriolopathy and salt sensitivity (18). The present findings suggest that purinergic signals may be one of the causative agents leading to preglomerular vessel hypertrophy. These data thus indicate that activation of vascular and glomerular purinergic receptors as well as on resident platelets may be an important part of the early

cascade of events that contribute to renal mesangial cell transformation and vascular hypertrophy that occur in ANG II-dependent hypertension. Accordingly, interruption of these early phases may have important relevance in preventing further progression.

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Fig. 1.

Immunolocalization of α -smooth muscle actin in paraffin-embedded rat kidney sections, α -actin (DAB chromogen in brown) is observed constitutively in the muscular layer of arterioles and pathologically in mesangial cells (A), sham-operated rat. B and C: glomeruli + arteriole, rat receiving ANG II infusion. D: rats receiving both ANG II infusion and clopidogrel.

A α -smooth muscle actin expression

B α -smooth muscle actin expression

Fig. 2.

Extent of mesangial cell transformation characterized as percentage of glomerular area expressing α -smooth muscle actin. CLOP, clopidogrel; PPADS, purinergic antagonist pyridoxal-phosphate-6-azophenyl-z',4'-disulphonic acid tetrasodium. * $P < 0.05$ vs. sham. † $P < 0.05$ vs. ANG II.

Fig. 3. Afferent arteriole wall thickness characterized as the width of the muscular layer of a circular section of an afferent arteriole. * $P < 0.05$ vs. sham. $\dagger P < 0.05$ vs. ANG II.

Fig. 4. Immunolocalization of proliferating cell nuclear antigen (PCNA) in paraffin-embedded rat kidney sections, PCNA (Fast-red chromogen in red) is observed in the nuclei of proliferating cells (A), tubular and interstitial area of sham-operated rat. B: tubular and interstitial area of ANG II-infused rat. C: tubular and interstitial area of rats receiving both ANG II infusion and clopidogrel. D: glomerulus of sham-operated rat. E: glomerulus of ANG II-infused rat. F: glomerulus of rats receiving both ANG II infusion and clopidogrel.

Fig. 5. Immunolocalization of macrophage-specific antigen (ED-1) in a paraffin-embedded rat kidney section, ED-1 (DAB chromogen in brown) is observed in the cytoplasm of infiltrating macrophages (A), cortical interstitium of sham-operated rat. B: cortical interstitium of ANG II-infused rat. C: cortical interstitium of rats receiving both ANG II infusion and clopidogrel.

Fig. 6. Density of renal cortical cells staining positively for PCNA (A), tubular cells. B: interstitial cells. C: glomerular cells. * $P < 0.05$ vs. sham. † $P < 0.05$ vs. ANG II + CLOP.

Fig. 7. Density of renal cortical cells staining positively for macrophage-specific antigen ED-1 (A), interstitial cells. B: glomerular cells. * $P < 0.05$ vs. sham. † $P < 0.05$ vs. ANG II + CLOP.

Fig. 8.

A: renal interstitial fluid concentrations in sham-operated rats and rats receiving ANG II infusion for 14 days. **B:** relationship between renal cortical interstitial fluid (RIF) ATP and mean arterial pressure (MAP) in control and ANG II-infused rats. * $P < 0.05$ vs. sham; $R^2 = 0.45$ ($P < 0.05$).

Table 1
Baseline SBP and SBP, and urinary albumin excretion at 14 days of ANG II infusion or sham operation

	SBP Baseline, mmHg	SBP Final, mmHg	U _{alb} V, mg/day
<i>Protocol I</i>			
Sham	123±8	123±4	0.13±0.02
ANG	126±1	193±7 ^{*†}	9.67±2.03 [‡]
ANG + Clopidogrel	124±7	193±4 ^{*†}	7.45±1.16 [‡]
<i>Protocol II</i>			
Sham	115±4	117±3	0.50±0.10
ANG	115±3	199±6 ^{*†}	7.90±2.31 [‡]
ANG + PPADS	122±5	195±7 ^{*†}	5.02±1.63 [‡]

Results are expressed as means ± SE. SBP, systolic blood pressure; U_{alb}V, daily urinary albumin excretion rate; PPADS, purinergic antagonist pyridoxal-phosphate-6-azophenyl-z',4'-disulphonic acid tetrasodium.

^{*} $P < 0.05$ vs. sham rats.

[†] $P < 0.05$ vs. baseline SBP.

[‡] $P < 0.05$ vs. sham rats.

Table 2
Cortical renal cell proliferation and inflammation in PPADS-treated rats

<i>Protocol II</i>	ED1_{int}⁺, cell/mm²	ED1_{glom}⁺, cell/glom	PCNA_{tub}⁺, cell/mm²	PCNA_{int}⁺, cell/mm²	PCNA_{glom}⁺, cell/glom
Sham	51±7	1.4±0.2	24±3	16±4	1.0±0.1
ANG	102±12 [*]	1.2±0.1	91±14 [*]	48±12 [*]	3.0±0.6 [*]
ANG + PPADS	52±3 [†]	1.3±0.1	49±7 [†]	23±6 [†]	1.7±0.4 [*]

Results are expressed as means ± SE. ED1_{int}⁺, density of interstitial cells staining positively for the macrophage-specific antigen ED-1; ED1_{glom}⁺, density of glomerular cells staining positively for ED-1; PCNA_{tub}⁺, density of tubular cells staining positively for the proliferating cell nuclear antigen PCNA; PCNA_{int}⁺, density of interstitial cells staining positively for PCNA; PCNA_{glom}⁺, density of glomerular cells staining positively for PCNA.

^{*} $P < 0.05$ vs. sham.

[†] $P < 0.05$ vs. ANG.