P2X₁ Receptor-Mediated Vasoconstriction of Afferent Arterioles in Angiotensin II–Infused Hypertensive Rats Fed a High-Salt Diet

Edward W. Inscho, Anthony K. Cook, Andrea Clarke, Shali Zhang, Zhengrong Guan

Abstract—Experiments tested the hypothesis that P2 receptor reactivity is impaired in angiotensin (Ang) II hypertensive rats fed an 8% NaCl diet (Ang II+HS). Juxtamedullary afferent arteriolar autoregulatory behavior was determined over a pressure range of 65 to 200 mm Hg. Arteriolar responsiveness to P2X₁ (β,γ-methylene ATP) or P2Y₁ receptor (uridine triphosphate) activation was determined in vitro. Systolic blood pressure averaged 126±3 and 225±4 mm Hg in control and Ang II+HS rats, respectively (P<0.05). In control kidneys, β,γ-methylene ATP (10⁻⁸ to 10⁻⁴ mol/L) reduced arteriolar diameter by 8±3%, 13±5%, 19±5%, 22±6%, and 24±9%, respectively, whereas uridine triphosphate reduced diameter by 2±1%, 2±2%, 9±3%, 37±7%, and 58±7%. Autoregulation was markedly blunted in Ang II+HS kidneys, with arteriolar diameter remaining essentially unchanged when perfusion pressure increased to 200 mm Hg compared with a 40±2% decline in diameter observed in normal kidneys over the same pressure range (P<0.05). P2X₁ receptor–mediated vasoconstriction was significantly attenuated in Ang II+HS kidneys. β,γ-Methylene ATP reduced arteriolar diameter by 1±1%, 3±2%, 6±1%, 9±3%, and 7±1%, respectively (P<0.05), versus control rats. Similar patterns were noted when hypertensive perfusion pressures were used. Uridine triphosphate–mediated responses were unchanged in Ang II+HS rats compared with control, indicating preservation of P2Y₂ receptor function. Ang II+HS blunted P2X₁-mediated increases in intracellular Ca²⁺ concentration in preglomerular smooth muscle cells. Therefore, Ang II+HS rats exhibit attenuated afferent arteriolar responses to P2X₁ receptor stimulation. These data support the hypothesis that P2X₁ receptors are important for pressure-mediated autoregulatory responses. Impairment of P2X₁ receptor function may explain the hypertension-induced decline in renal autoregulatory capability. (Hypertension. 2011;57:780-787.) • Online Data Supplement

Key Words: P2 receptors ■ Ang II hypertension ■ Ca²⁺ signaling ■ autoregulation ■ UTP ■ ATP ■ adenosine

Previous work indicates that β,γ-methylene ATP (P2X₁) receptors contribute importantly to pressure-mediated autoregulatory vasoconstriction of afferent arterioles. P2X₁ receptors are ligand-gated channels that stimulate afferent arteriolar vasoconstriction through calcium (Ca²⁺) influx-dependent mechanisms. Uridine triphosphate (UTP; P2Y₁) receptors also stimulate vasoconstriction but do so mainly through release of Ca²⁺ from intracellular stores. Inactivation of P2X₁ receptors blocks pressure-mediated autoregulatory vasoconstriction, suggesting that increases in transmural pressure lead to autoregulatory vasoconstriction by ATP-dependent P2X₁ receptor activation.

Renal perfusion is primarily controlled by agonist-induced and pressure-mediated adjustments in preglomerular resistance. Regulation of preglomerular resistance is central to the maintenance of glomerular capillary pressure. Afferent arterioles are the main vascular elements responsible for pressure-mediated autoregulatory adjustments in preglomerular resistance to regulate renal blood flow and glomerular filtration rate. Autoregulatory reactivity is attenuated in several forms of hypertension, including the angiotensin (Ang) II–infused model; however, the mechanisms responsible for blunted autoregulatory behavior are unclear. Along with a reduced response to pressure, afferent arterioles from hypertensive rats also show blunted vasoconstrictor responses to P2X₁ receptor activation. Therefore, reduction of P2X₁ receptor reactivity strongly correlates with blunted autoregulatory efficiency.

This study was performed to extend the previous report to determine whether afferent arteriolar autoregulatory behavior and P2X₁ receptor–mediated vasoconstriction could be eliminated in a model of salt-sensitive hypertension that includes increased dietary salt. For these studies, we combined chronic subpressor infusion of Ang II with a high-salt diet. We examined autoregulatory behavior, afferent arteriolar P₁ and P₂ receptor reactivity, and preglomerular smooth muscle cell Ca²⁺ signaling. Results indicate blunted but not abolished (passive vascular behavior) autoregulatory responsiveness, coincident with reduced P2X₁ receptor reactivity and Ca²⁺ signaling.
Methods

Please see the online Data Supplement at http://hyper.ahajournals.org for an expanded description of the methods.

Animals

Experiments were performed on male Sprague-Dawley rats weighing 225 to 250 g. Some rats received chronic Ang II infusion (60 ng/min; 14 days) by osmotic minipump, as described.11,12 Ang II–infused rats averaged 14.9±4.1 g in Ang II–HS rats compared with 357±15 g in controls (P<0.05). Further, experimentally induced hypertension was performed as described.5,11 Cells were exposed to physiological salt solution (PSS) containing ATP, β,γ-methylene ATP, or UTP (10 μmol/L) or to adenosine (1 and 100 μmol/L). Ca2+ responses were evaluated by averaging the magnitude of the peak [Ca2+] at achieved during 200 seconds of agonist exposure.

Western Blot Analysis of P2X1 Receptor Expression in Kidney

Homogenates of renal cortex, medulla, and preglomerular microvessels were prepared using kidneys from separate control or Ang II–HS rats, as described.11,15

Results

Ang II+HS treatment increased systolic blood pressure (225±4 mm Hg; P<0.05) significantly compared with normotensive control rats (126±3 mm Hg; Table; Figure S1, available in the online Data Supplement at http://hyper.ahajournals.org). Systolic blood pressure increased similarly in all of the groups of Ang II+HS rats prepared for different agonist protocols (Table). Body weights were similar across all of the groups and averaged ~230 g before initiation of Ang II infusion and HS diet. Ang II+HS rats gained weight during the 14-day treatment period but not as rapidly as normotensive controls. By day 14, body weights averaged 292±7 g in Ang II+HS rats compared with 357±15 g in controls (P<0.05).

Table. Mean Systolic Blood Pressure Data on Days 0, 7, and 14

<table>
<thead>
<tr>
<th>Treatment Group (n)</th>
<th>Day 0</th>
<th>Day 7</th>
<th>Day 14</th>
</tr>
</thead>
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<tr>
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<td>128±4</td>
<td>125±8</td>
<td>126±3</td>
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<tr>
<td>ATP (7)</td>
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<td>206±16</td>
<td>234±6</td>
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<tr>
<td>β,γ-methylene ATP (7)</td>
<td>115±5</td>
<td>186±24</td>
<td>232±8</td>
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<td>UTP (8)</td>
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<tr>
<td>Adenosine (7)</td>
<td>123±4</td>
<td>206±4</td>
<td>227±7</td>
</tr>
</tbody>
</table>

Renal Microvascular Smooth Muscle Cell Isolation

Preglomerular microvessels were isolated from separate rats as described.11 Dispersed cells were collected and loaded with the calcium-sensitive fluorescent probe, fura 2-acetylmethyl ester (10.0 μmol/L) for study. Measurement of intracellular calcium concentration ([Ca2+]i) in single preglomerular smooth muscle cells was performed as described.11,12 Cells were exposed to physiological salt solution (PSS) containing ATP, β,γ-methylene ATP, or UTP (10 μmol/L) or to adenosine (1 and 100 μmol/L). Ca2+ responses were evaluated by averaging the magnitude of the peak [Ca2+]i, achieved during 200 seconds of agonist exposure.

Afferent Arteriolar Responses

Videomicroscopy experiments were conducted in vitro using the blood-perfused juxtanodillary nephron technique, as described.13,14 Sustained afferent arteriolar diameter was calculated from the average of measurements made during the final 2 minutes of each treatment period. For the autoregulatory portion, arteriolar diameters were measured during step (15-mm Hg) increases in renal perfusion pressure from 65 to 170 mm Hg in successive 5-minute periods11 followed by a 10-minute recovery period (100 mm Hg). A separate group of kidneys underwent an autoregulatory profile that ranged from 95 to 200 mm Hg in successive 5-minute periods11 to 170 mm Hg to reflect the hypertensive pressures. Then arteriolar diameters compared with normotensive controls.

Afferent arteriolar response to increasing perfusion pressure in kidneys of normotensive (open symbols) and Ang II+HS (closed symbols) rats. Autoregulatory profiles ranging from 65 to 170 mm Hg are depicted by circular symbols, whereas profiles generated from 95 to 200 mm Hg are depicted by square symbols. A. The actual diameter responses expressed in microns. B. The same data normalized as a percentage of the starting diameter for each group (percentage of control). Data points represent the mean±SE. *Significant difference (P<0.05) from the baseline diameter at 100 mm Hg; #Significant difference (P<0.05) from the response of normotensive controls; n=the number of afferent arterioles studied.
200 mm Hg in Ang II+HS (n=4) rats had no significant effect on afferent diameter. The pressure-mediated vasoconstrictor response was significantly blunted in hypertensive Ang II+HS kidneys compared with normotensive controls.

**Afferent Arteriolar Responses to ATP**

Responses to ATP are presented in Figure 2A. Baseline diameters were similar between the control and Ang II+HS groups, averaging 15.7±0.9 μm (n=6) and 14.9±0.5 μm (n=7), respectively. In normotensive rats, superfusion with ATP changed arteriolar diameter to 101±1%, 94±1%, 86±2%, 76±2%, and 71±5% of control diameter for ATP concentrations of 10⁻⁸ to 10⁻⁴ mol/L. Ang II+HS blunted vasoconstrictor responses to ATP, because diameter decreased only to 98±1%, 95±1%, 87±3%, 81±4%, and 85±5% of control over the same concentration range. These data demonstrate that arteriolar responses to ATP are attenuated in Ang II+HS kidneys.

**Afferent Arteriolar Responses to P2X₁ Receptor Activation**

Autoregulatory behavior is linked to P2X₁ receptor activation. Blunted reactivity to ATP could reflect reduced P2X₁ receptor reactivity. Afferent arteriolar responses to the P2X₁ agonist, β,γ-methylene ATP are presented in Figure 2B. Baseline diameters were similar between control and Ang II+HS groups at a perfusion pressure of 100 mm Hg, averaging 15.8±0.2 μm (n=5) and 16.7±0.3 μm (n=7), respectively. In control rats, β,γ-methylene ATP decreased arteriolar diameter to 92±2%, 87±5%, 81±6%, 78±6%, and 76±9% of control diameter for concentrations of 10⁻⁸ to 10⁻⁴ mol/L. Ang II+HS markedly blunted the vasoconstrictor responses. β,γ-Methylene ATP decreased diameter to just 99.0±0.4%, 97.0±2.0%, 94.0±1.0%, 91.0±3.0%, and 93.0±1.0% of control over the same concentration range. These data indicate that afferent arteriolar responses to P2X₁ receptor stimulation are greatly attenuated in kidneys from Ang II+HS-treated hypertensive rats.

We also examined P2X₁ receptor reactivity in separate kidneys perfused at the hypertensive pressure (170 mm Hg; Figure 2B). Consistent with intact autoregulatory behavior, baseline diameters were significantly lower in the control kidneys, at 12.5±0.2 μm (n=7), compared with Ang II+HS kidneys, at 14.9±0.3 μm (P<0.05; n=5). In control rats at 170 mm Hg, β,γ-methylene ATP decreased arteriolar diameter to 95±1%, 92±1%, 88±2%, 85±2%, and 83±1% of control diameter for concentrations of 10⁻⁸ to 10⁻⁴ mol/L. Ang II+HS still blunted the vasoconstrictor responses such that β,γ-methylene ATP decreased diameter to just 99±1%, 98±2%, 94±2%, 92±4%, and 88±5% of control over the same concentration range. Collectively, these data indicate that afferent arteriolar responses to P2X₁ receptor stimulation are greatly attenuated in kidneys from Ang II+HS-treated hypertensive rats perfused at normotensive or hypertensive pressures.

**Afferent Arteriolar Responses to P2Y Receptor Activation**

Afferent arterioles exhibit significant vasoconstrictor responses to the P2Y₂ agonists UTP and adenosine 5'-o-(3-thio triphosphate) (ATP-γ-S). Therefore, studies were performed to determine the effect of Ang II+HS hypertension on arteriolar responses to UTP (Figure 3A). Baseline diameters at 100 mm Hg were similar between control and Ang II+HS groups, averaging 15.5±0.4 μm (n=7) and 15.2±0.8 μm (n=8), respectively. In control rats, UTP decreased arteriolar diameter to 98±1%, 98±2%, 91±3%, 63±7%, and 42±7% of control for concentrations of 10⁻⁸ to 10⁻⁴ mol/L. In Ang II+HS kidneys, arteriolar diameter declined to 100±2%, 97±3%, 89±3%, 75±7%, and 61±9% of control over the same concentration range. The magnitudes of these changes are similar to responses in control kidneys. These data indicate that afferent arteriolar responses to P2Y₂ receptor stimulation are unchanged in Ang II+HS hypertension.

We also examined the responses to UTP in kidneys perfused at hypertensive pressure (Figure 3A). Baseline diameters were significantly smaller in control kidneys compared with Ang II+HS groups, averaging 13.0±0.2 μm (n=6) and 15.5±0.1 μm (n=4), respectively, consistent with intact autoregulation. UTP reduced diameter similarly between the control and Ang II+HS groups at 170 mm Hg, but overall the maximum responses were smaller at hypertensive pressures than at normotensive pressures. UTP decreased arteriolar diameter to 97±1%, 95±2%, 90±3%, 83±4%,
75±5% of control for concentrations of 10⁻⁸ to 10⁻⁴ mol/L in control kidneys. In Ang II+HS kidneys, arteriolar diameter averaged 98±1%, 96±2%, 89±3%, 79±2%, and 71±3% of control over the same concentration range. These data indicate that afferent arteriolar responses to P2Y₂ receptor stimulation are unchanged in Ang II+HS hypertension compared with normotensive kidneys.

Afferent Arteriolar Responses to Adenosine Receptor Activation

Adenosine influences afferent arteriolar diameter through activation of A₁ receptors and is postulated to mediate tubuloglomerular feedback responses. Experiments were performed to compare arteriolar responses to adenosine in control and Ang II+HS rats. Baseline diameters were similar between the control (15.1±0.9 μm; n=6) and Ang II+HS (15.1±0.8 μm; n=7) groups (Figure 3B). In control rats, adenosine changed arteriolar diameter to 96±1%, 91±2%, 85±4%, 93±2%, and 106±4% of baseline for concentrations of 10⁻⁸ to 10⁻⁴ mol/L. Ang II+HS had only slight effects on the responses to adenosine. In these kidneys, the same concentrations of adenosine changed arteriolar diameter to 99±1%, 97±2%, 93±1%, 94±1%, and 100±2% of control. Only the response at 10⁻⁶ mol/L in Ang II+HS was statistically reduced (P=0.045) compared with control arterioles.

The lack of response to P2X₁ receptor stimulation cannot be attributed to complete loss of contractile ability by afferent arterioles, because 55 mmol/L of KCl treatment evoked detectable (Figure 5A). In control cells, [Ca²⁺]i averaged 40±6 nmol/L (22 cells per 8 rats), then declined to a lower plateau.

Ca²⁺ Responses to β₃,γ-Methylene ATP and ATP

P2 receptors use [Ca²⁺], as a major signaling pathway mediating vasoconstriction. Experiments were performed to compare the Ca²⁺ signaling responses of preglo- merular smooth muscle cells isolated from control and Ang II+HS rats (Figures 4 and 5). As shown by the typical traces in Figure 4A, the P2X₁ agonist β₃,γ-methylene ATP (10 μmol/L) evoked a rapid, biphasic increase in [Ca²⁺], in

cells from normotensive rats, and that response was markedly blunted in cells from Ang II+HS rats. Peak [Ca²⁺], responses increased by 517±132 nmol/L in control cells (12 cells per 4 rats) compared with 73±11 nmol/L in Ang II+HS cells (18 cells per 7 rats; Figure 5B). Similarly, [Ca²⁺], responses evoked by the dual P2X/P2Y agonist ATP (10 μmol/L) were significantly reduced in Ang II+HS cells compared with controls (Figure 4B). ATP stimulated a muted increase in [Ca²⁺], in Ang II+HS cells, averaging 189±34 nmol/L (34 cells per 11 rats) compared with 428±68 nmol/L in control cells (68 cells per 10 rats; Figure 5B).

Ca²⁺ Responses to UTP, Adenosine, and KCl

Renal microvessels express P2X₁, P2Y₂ receptors, and adenosine A₁ and A₂ receptors. Experiments were performed to determine the impact of Ang II+HS hypertension on [Ca²⁺], induced by UTP (P2Y₂ agonist), adenosine, and KCl in preglo- merular smooth muscle cells. In controls, ATP stimulated a sharp, biphasic increase in [Ca²⁺], (Figure 4C and 5B). This response peaked rapidly, averaging 722±123 nmol/L (22 cells per 8 rats), then declined to a lower plateau. Spontaneous Ca²⁺ oscillations were frequently observed during the plateau phase. In Ang II+HS cells, [Ca²⁺], responses were not significantly altered and averaged 552±114 nmol/L (30 cells per 9 rats).

In contrast, adenosine (1 μmol/L) responses were not detectable (Figure 5A). In control cells, [Ca²⁺], averaged 48±6 nmol/L (12 cells per 3 rats) during control conditions and 49±6 nmol/L during exposure to adenosine. Similarly, in cells from Ang II+HS kidneys, [Ca²⁺], averaged 40±4 nmol/L (12 cells per 3 rats) during control conditions and 40±4 nmol/L during adenosine treatment. As a positive control to verify reactivity in adenosine-treated cells, a second agonist exposure period was imposed using 10 μmol/L of ATP. The same cells that failed to show adenosine-mediated Ca²⁺ responses exhibited marked increases in [Ca²⁺]. These positive control ATP responses produced average increases of 452±130 nmol/L compared with 134±37-nmol/L cells from control and Ang II+HS cells, respectively (Figure 5A; P<0.05). Similarly, responses to a 100-fold higher concentration of adenosine (10 μmol/L) were also not detectable. In control cells, [Ca²⁺], averaged...
second positive control, we also exposed separate cells to 90 mmol/L of KCl to assess Ca2+ responses to nonreceptor-dependent stimuli. KCl responses averaged 171±46 nmol/L (23 cells per 11 rats) in control cells and 140±38 nmol/L (24 cells per 8 rats) in Ang II+HS cells (Figure 5B).

Western Analysis for Changes in P2X1 Receptor Expression

Preglomerular microvessels from both normotensive control and Ang II+HS rats exhibited strong P2X1 receptor protein expression (Figure S2). Densitometric analysis revealed that protein expression was similar in preglomerular microvessels from control rats (40±2 arbitrary densitometry units; n=6) versus microvessels from Ang II+HS rats (37±4 arbitrary density units; n=6). P2X1 receptor protein expression was undetectable in the renal cortex and renal medulla in both normotensive and hypertensive settings.

Discussion

Autoregulatory behavior is essential for maintaining stable renal blood flow, glomerular capillary pressure, and glomerular filtration rate. Autoregulatory impairment is potentially harmful to the kidney by limiting the microvasculature’s ability to protect glomeruli from harmful hypertensive pressures.8,9,20 Autoregulatory efficiency is reduced in models of
reduced renal mass,21 Goldblatt hypertension,9 Dahl salt-sensitive hypertension,10 Nω-nitro-L-arginine methyl ester hypertension,7 and Ang II hypertension,11–13 as well as high dietary salt.22 Although impairment of autoregulation is recognized, the mechanism(s) responsible remain unclear. The study examines afferent arteriolar reactivity to postulated autoregulatory messenger molecules and receptors to link known autoregulatory signaling pathways with functional impairment in Ang II+HS hypertension.

Autoregulation represents the combined influences of myogenic and tubuloglomerular feedback mechanisms.6,16,20 ATP and adenosine are extracellular messenger molecules postulated to mediate autoregulatory adjustments in preglomerular resistance. ATP and adenosine vasoconstrict afferent arterioles by activating P2 and P1 (A1) receptors, respectively.1,16,19 The P2 receptor family is divided into 2 structurally and functionally distinct subfamilies, classified P2X and P2Y.17–19 P2X receptors are ligand-gated channels that function as nonselective cation channels.17–19 P2Y receptors are metabotropic, G protein–coupled receptors that signal largely through the release of calcium from intracellular stores.18,19 In contrast, P1 receptors are activated by extracellular adenosine and are divided into 3 families classified as A1, A2, and A3 receptors.18,19 A1 and A2 receptors are also G protein–coupled receptors that signal largely by inhibiting or stimulating adenyl cyclase activity, respectively.18,19 Consequently, A1 receptor activation inhibits cAMP production and evokes vasoconstriction, whereas A2 receptor activation stimulates cAMP accumulation and evokes vasorelaxation. A1 receptors appear to signal through modulation of [Ca2+]i. Accordingly, we examined the impact of Ang II+HS on afferent arteriolar autoregulatory behavior and reactivity to P2 and P1 receptor activation.

Previous work supports the hypothesis that P2X1 receptor activation is essential for transducing increases in perfusion pressure with afferent arteriolar vasoconstriction to produce autoregulatory resistance adjustments.1,2 Pharmacological blockade of P2 receptors with nonselective blockers and with P2X1 receptor-selective antagonists uniformly inhibits autoregulatory behavior.1,2,13,23 Global deletion of P2X1 receptors inhibits autoregulation in P2X1 knockout mice compared with wild-type littermates.2 Accordingly, decreased afferent arteriolar reactivity to P2 receptor activation was implicated in the functional impairment of autoregulation in Ang II+HS rats. ATP is postulated as the endogenous ligand for renal microvascular P2 receptors, so we determined the arteriolar response to P2 receptor stimulation with ATP. Results indicate that arteriolar responsiveness to P2 receptor activation by ATP is blunted in Ang II+HS rats compared with normotensive rats similar to Ang II hypertension alone.11 This impairment is consistent with impaired autoregulation but does not differentiate which P2 receptor subtype(s) are involved. Interestingly, this impairment is not exacerbated by the addition of high salt to the hypertensive setting.

Experiments were conducted to assess the impact of Ang II+HS on afferent arteriolar reactivity to P2X1 receptor activation. We selected β,γ-methylene ATP as an agonist with high selectivity for P2X1 receptors and some ability to bind to P2X3 receptors.17 Current and previous work clearly indicate strong P2X1 receptor expression by the preglomerular microvasculature,11,24–26 but P2X3 receptor expression by afferent arterioles is not established. In preliminary experiments, we could not detect P2X3 receptor protein by Western blot in preglomerular microvessels of normotensive animals (data not shown). Lewis and Evans26 report “barely detectable” P2X3 immunostaining of small intrarenal arteries but did not report on afferent arterioles. Turner et al25 did not detect P2X3 immunostaining in renal arterioles. Finally, Harhun et al27 detects the PCR product for P2X1 and P2X4 while not detecting gene expression for P2X2, P2X4, P2X5, or P2X7 receptors. These data suggest that P2X1 receptors are the primary mechanisms by which β,γ-methylene ATP vasoconstricts afferent arterioles. In the current report, arteriolar vasoconstriction to β,γ-methylene ATP was blunted in kidneys from Ang II+HS rats compared with normotensive controls. Blunted reactivity was observed regardless of whether the kidneys were perfused at normotensive or hypertensive pressures, suggesting an inherent decline in P2X1 receptor signaling. These same kidneys also exhibited impaired autoregulatory behavior, supporting the hypothesis that P2X1 receptors mediate pressure-dependent autoregulation. These data further suggest that impaired autoregulation in Ang II+HS rats reflects impaired P2X1 receptor signaling.

Afferent arterioles also respond to the P2Y2 receptor agonist UTP and to the P1 receptor agonist adenosine. Arteriolar responses to these agonists were essentially unchanged in Ang II+HS rats compared with normotensive controls despite impaired autoregulation. Interestingly, P2Y2–mediated vasoconstriction was uniformly blunted in control and Ang II+HS kidneys when perfused at hypertensive pressures. The mechanism for this remains unclear, but because the decline in function was similar in both the normotensive and hypertensive groups, it cannot explain the decline in autoregulatory efficiency. Compared with a previous study with Ang II hypertensive rats on a normal salt diet,11 the response to adenosine was attenuated at the 1.0 μmol/L concentration. The importance of this is unclear, but reduced adenosine reactivity cannot explain hypertension-induced impairment of autoregulatory efficiency, because adenosine-reactivity was normal in Ang II hypertension alone, where autoregulation was clearly impaired. The addition of salt to the hypertensive sequence may have a direct effect on adenosine receptor expression/function independent of blood pressure. Overall, these data support the postulate that autoregulation is mediated primarily by P2X1 receptors with P2Y2 receptors or P1 receptors not making major contributions to this pressure-dependent response.

Exactly how hypertension impairs autoregulatory behavior is unclear. Chronic P2 receptor blockade in vivo prevents mesangial cell proliferation and vascular thickening of afferent arterioles of Ang II hypertensive rats, despite having no detectable effect on blood pressure, renin, or renal Ang II content.28 Recent studies suggest that local inflammation may influence arteriolar function and inhibit autoregulatory efficiency.12 In those studies, simultaneous treatment with the broad-spectrum anti-inflammatory agent pentosan polysulfate provided protection against autoregulatory impairment in Ang II hypertensive rats without changing the progression or...
magnitude of the hypertension. Pentosan polysulfate also preserved arteriolar reactivity to P2X, stimulation with β,γ-methylene ATP. In other studies, pentosan polysulfate is renoprotective in reduced renal mass hypertension, and it restored glomerular hemodynamics, independent of arterial hypertension. "Transient exposure to chronic Ang II infusion confers greater salt sensitivity and interstitial inflammation, suggesting that induction of Ang II hypertension elicits inflammation in the kidney that negatively impacts microvascular function. They also suggest that inflammation is linked to renal injury imposed by adding salt to a hypertensive setting. Collectively, it is possible that initiation of intrarenal inflammation is directly linked to impaired P2X, receptor function.

We reported previously that P2X, receptor expression appears normal in kidneys from Ang II hypertensive rats. The current report confirms and extends that observation by indicating that P2X, receptor expression was also unchanged when salt was combined with Ang II–infused hypertension. The explanation for impaired P2X, receptor activity in the face of relatively normal P2X, receptor expression is unclear. Vial and coworkers reported that P2X receptors are influenced by their association with lipid rafts. Disruption of lipid rafts renders P2X, receptors dysfunctional. It is conceivable that hypertension, with or without excess salt, could alter the microvascular lipid raft environment and lead to reduced P2X, receptor functionality despite normal levels of P2X, receptor protein expression. Similarly, it is possible that perivascular inflammation could negatively impact P2X, receptor association with lipid rafts and reduce P2X, receptor activity. Alternatively, P2X, receptor trafficking could be disrupted in hypertension and lead to reduced P2X, receptor expression in the sarcolemma. A fourth possibility is that signaling downstream of P2X, receptor activation is compromised. Preglomerular microvascular P2X, receptors signal through calcium and rho-kinase activity. The inhibition blocks autoregulation and P2X, receptor-mediated vasoconstriction while having a more modest effect on P2Y, receptor-mediated vasoconstriction. More extensive studies are required to help resolve these possibilities.

P2X, receptor-dependent Ca++ signaling is impaired in Ang II hypertensive rats, so alterations in downstream signaling pathways/events are potential explanations for the blunted autoregulatory and P2X, receptor-mediated events. Consistent with previous findings, Ca++ signaling responses for ATP and P2X, receptor activation are markedly impaired, but the degree of impairment is not notably different from that seen with Ang II infusion alone. Importantly, blunted Ca++ signaling responses are unique to the P2X, receptor, because responses to UTP-dependent P2Y, receptor activation and KCl depolarization were unchanged. In addition, the response to 10 μmol/L of ATP, which activates P2X and P2Y receptors, was also significantly blunted. This may reflect signaling loss of the P2X Ca++ component and retention of the P2Y portion of the overall ATP-dependent response. These data support a key role for P2X, receptor activation and ligand-gated Ca++ signaling in mediating autoregulatory vasoconstriction. Importantly, 1 and 100 μmol/L of adenosine did not increase [Ca++], in cells that yielded robust [Ca++], responses to ATP. Given that afferent arteriolar autoregulation is Ca++ dependent, lack of a Ca++ response to adenosine calls into question the suitability of adenosine as a major signaling molecule mediating overall autoregulatory control.

This study demonstrates that Ang II+HS hypertension significantly impairs autoregulation of rat juxtaglomerular afferent arterioles. Coincident with this impairment is a decline in afferent arteriolar reactivity to ATP and to P2X, receptor activation, whereas reactivity to the P2Y, agonist UTP or to the P1 receptor agonist adenosine is essentially unchanged. Importantly, autoregulatory impairment was manifested at perfusion pressures as high as 200 mm Hg, eliminating the possibility that these hypertensive rats merely operate with a right-shifted pressure-flow relationship. In addition, refractory arteriolar reactivity to P2X, receptor stimulation was apparent at both normotensive and hypertensive pressures, whereas KCl-mediated vasoconstriction was clearly evident. Decreased reactivity to P2X, receptor activation cannot be attributed to decreased P2X, receptor expression, because Western blot analysis reveals no detectable difference in P2X, receptor protein levels in preglomerular vascular tissue, but Ca++ signaling responses to P2X, activation are markedly blunted. This study extends previous reports of impaired autoregulatory behavior in hypertensive models by implicating decreased afferent arteriolar reactivity to P2X, receptor activation as a causal link.

Perspectives

The impact of hypertension on renal microvascular function is an important variable in the progression to hypertensive renal injury. Efficient autoregulatory control protects healthy kidneys from barotrauma that comes from acute elevations in renal perfusion pressure. Hypertension, however, brings chronic elevations in blood pressure that impairs microvascular autoregulatory function and microvascular reactivity to some vasoactive agents and leads to glomerular hypertrophy and injury. Resolving the mechanisms that underlie this loss of function will facilitate our ability to design therapeutic interventions that will prevent renal decline early in the hypertensive progression and may permit reversal of existing impairment. This would indeed provide an improved quality of life and prolong normal renal function in afflicted patients.

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Disclosures

None.

References


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SUPPLEMENTAL METHODS

Animals
Experiments were performed on male Sprague-Dawley rats (Charles River Labs; Raleigh, North Carolina) weighing 225-250 g. Some rats received a chronic Ang II infusion (60 ng/min; 14 days) administered by osmotic minipump (Model 2002; Durect Corporation, Cupertino, CA) implanted subcutaneously in isoflurane anesthetized rats, as previously described \(^1,^2\). Ang II infused rats were fed an 8% salt diet (TD.92012; Harlan-Teklad Laboratories, Madison, WI) during the Ang II infusion period. Systolic blood pressure (SBP; tail cuff plethysmography (IITC Model 65-12, Woodland Hills, CA)) and body weights were measured every three to four days. Animals were treated according to the NIH Guide for the Care and Use of Laboratory Animals using procedures approved by the Medical College of Georgia Institutional Animal Care and Use Committee.

Afferent Arteriole Responses
Videomicroscopy experiments were conducted \emph{in vitro} using the blood-perfused juxtamedullary nephron technique \(^2,^3\). For each experiment, two identically prepared animals were anesthetized with sodium pentobarbital (50 mg/kg; i.p.). Perfusate blood was collected from a blood donor and the kidney donor rat and prepared, as previously described \(^2,^3\).

Arteriolar diameters were recorded at 12-second intervals. Sustained afferent arteriolar diameter was calculated from the average of measurements made during the final 2 minutes of each treatment period. Following a 15-min equilibration period, baseline afferent arteriolar diameters were measured. Each kidney underwent an autoregulatory profile followed by a concentration response determination for P1 and P2 receptor agonists. For the autoregulatory portion, arteriolar diameters were measured during step (15 mmHg) increases in renal perfusion pressure from 65 to 170 mmHg in successive 5-minute periods \(^1\) followed by a 10 min recovery period (100 mmHg). A separate group of kidneys underwent an autoregulatory profile that ranged from 95 to 200 mmHg to be sure that hypertensive pressures were covered. Then arteriolar responses to P1 or P2 agonists were determined at a perfusion pressure of 100mmHg. A separate group of kidneys underwent selected agonist reactivity studies in kidneys perfused at 170mmHg to determine if perfusion pressure alone altered reactivity. One agonist was used per kidney. Afferent arterioles were exposed to increasing concentrations of ATP, β, γ–methylene ATP, UTP or adenosine (10\(^{-8}\) to 10\(^{-4}\) mol/L), and diameter was monitored for 5 minutes/concentration.

Renal Microvascular Smooth Muscle Cell Isolation.
Preglomerular microvessels were isolated from separate groups of anesthetized rats as previously described \(^1\). The kidneys were perfused with physiological salt solution (PSS), and microvessels were separated from the rest of the cortex. Microvessels were dissected away from arterial segments and transferred to a digestion solution containing 0.4% albumin, 0.15% papain and 0.05% dithiothreitol in low-calcium PSS at 36.5°C. After 30-minutes, the mixture was gently tritiruated and centrifuged (500xg; 5 min.) to
collect dispersed cells. Cells were resuspended in 1.0 mL Dulbecco’s modified Eagle’s medium and loaded with the calcium-sensitive fluorescent probe, fura 2-acetoxyethyl ester (fura 2-AM; 10.0 μmol/L; Molecular Probes). An aliquot of cell suspension was transferred to a perfusion chamber mounted to the stage of an Olympus inverted microscope.

Measurement of intracellular calcium concentration ([Ca\textsuperscript{2+}]\textsubscript{i}) in single preglomerular smooth muscle cells was performed as described \textsuperscript{1, 4}. Cells were exposed to PSS containing ATP, β,γ-methylene ATP or UTP (10 μmol/L) which evokes pronounced P2 receptor-dependent [Ca\textsuperscript{2+}]\textsubscript{i} responses, or to adenosine (1 and 100 μmol/L). Ca\textsuperscript{2+} responses were evaluated by averaging the magnitude of the peak [Ca\textsuperscript{2+}]\textsubscript{i}; achieved during 200 seconds of agonist exposure.

**Western Blot Analysis of P2X\textsubscript{1} Receptor Expression in Kidney**

Homogenates of renal cortex, medulla and preglomerular microvessels were prepared from a separate group of kidneys from control or AngII+HS rats \textsuperscript{1, 5}. Samples were separated by electrophoresis, transferred to a nitrocellulose membrane incubated with primary P2X\textsubscript{1} polyclonal antibody (Alomone Labs Ltd., Jerusalem, Israel; 1:500), followed by secondary antibody (Santa Cruz Biotechnology, Santa Cruz, CA), and developed using enhanced chemiluminescence, as described \textsuperscript{1}.

**Data Analysis.**

Statistical comparisons within each series were made by one-way analysis of variance for repeated measurements combined with the Dunett’s multiple range test. Within-group comparisons of peak [Ca\textsuperscript{2+}]\textsubscript{i} with baseline [Ca\textsuperscript{2+}]\textsubscript{i} were analyzed using ANOVA for repeated measures. Across group comparisons of [Ca\textsuperscript{2+}]\textsubscript{i} responses were compared by one-way ANOVA and Dunnett’s post hoc tests. A probability value of \( P<0.05 \) was considered statistically significant.

**Materials**

Bicinchoninic acid protein assay kit was purchased from Pierce (Rockford, IL). Chemiluminescence kit was purchased from Amersham (Piscataway, NJ). Protease inhibitor cocktail was purchased from Roche Applied Science (Indianapolis, IN). All other reagents were purchased from Sigma Chemical Company (St. Louis, MO).

**References**


(2) Guan Z, Fuller BS, Yamamoto T, Cook AK, Pollock JS, Inscho EW. Pentosan polysulfate treatment preserves renal autoregulation in Ang II-infused hypertensive rats via normalization of P2X\textsubscript{1} receptor activation. *Am J Physiol Renal Physiol*. 2010;298:F1276-F1284.


**Figure S1.** Systolic blood pressure in control and AngII+HS rats. Blood pressure was measured every three to four days by tail cuff. Control rats are depicted by white circles and mean AngII+HS rats are shown by black circles. *represents a significant difference (P<0.05) between the control group and the combined AngII+HS groups.
Figure S2. Expression of P2X<sub>1</sub> receptor protein isoforms in rat preglomerular microvessels, renal cortex and medulla in normal and AngI+HS rats. Panel A presents a typical example revealing prominent bands for P2X<sub>1</sub> receptor protein at approximately 50 kDa in preglomerular microvessels and in the vas deferens, which was used as a positive control. Beta actin was used as a loading control. Panel B presents mean densitometry data for P2X<sub>1</sub> staining of preglomerular microvessels from normal (n=6 rats) and AngI+HS rats (n=6 rats). VD = Vas deferens; NT = normotensive controls; AngHS = AngI+HS.