Tyrosine Kinase Involvement in Renal Arteriolar Constrictor Responses to Angiotensin II

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Abstract—Experiments were performed to test the hypothesis that tyrosine kinase activity contributes to renal arteriolar contractile responses to angiotensin (Ang) II. Rats were subjected to short-term enalaprilat treatment to decrease endogenous Ang II formation before tissue was harvested for experiments with the in vitro blood-perfused juxtamedullary nephron technique. Acute surgical papillectomy was used to avoid the indirect afferent arteriolar effect of Ang II that arises through increased tubuloglomerular feedback sensitivity. Arteriolar lumen diameter responses to 1 and 10 nmol/L Ang II were monitored by videomicroscopic methods before and during treatment with various tyrphostin compounds: 100 μmol/L AG18 (broad-spectrum tyrosine kinase inhibitor), 100 nmol/L AG1478 (selective epidermal growth factor receptor tyrosine kinase inhibitor), or 100 μmol/L AG9 (inactive analog). Baseline afferent arteriolar lumen diameter averaged 23.5±1.2 μm and was not influenced by any tyrphostin. Ang II (10 nmol/L) decreased afferent diameter by 11.1±1.0 μm under untreated conditions, a response that was not altered by AG9 but significantly blunted by AG18 (34±9% inhibition) or AG1478 (52±8% inhibition). AG18 did not suppress afferent arteriolar contractile responses to membrane depolarization (20 to 55 mmol/L K+ bath). Efferent arteriolar baseline diameter averaged 24.1±0.8 μm and was unaltered by AG18 or AG1478; however, efferent diameter responses to 10 nmol/L Ang II were diminished 52±10% by AG18 and 51±13% by AG1478. These observations indicate that Ang II signaling in renal afferent and efferent arteriolar vascular smooth muscle is either mediated or modulated by tyrosine kinase activity, including that of the epidermal growth factor receptor tyrosine kinase. (Hypertension. 2001;37[part 2]: 569-573.)

Key Words: arterioles • angiotensin II • kinase • receptors

Although the G-protein–coupled angiotensin (Ang) II type 1 (AT1) receptor lacks intrinsic tyrosine kinase activity, Ang II binding to this receptor induces tyrosine phosphorylation of multiple signaling proteins. Tyrosine phosphorylation of phospholipase C (PLC)-γ has been reported to be necessary for Ang II–stimulated inositol triphosphate (IP3) formation, Ca2+ mobilization, and contraction of mesangial and aortic smooth muscle cells.1,2 Moreover, transactivation of the epidermal growth factor (EGF) receptor is evident in vascular smooth muscle within 1 to 2 minutes of Ang II exposure,3,4 consistent with the possibility that this process may contribute to the vasoconstrictor response to the peptide. Similarly, Ang II–induced activation of p38 mitogen-activated protein (MAP) kinase occurs within 5 minutes, and inhibition of p38 kinase activity reduces the contractile effect of Ang II on rat aortic rings.5 Nevertheless, Watts and colleagues6 have demonstrated dissociation of Ang II–induced tyrosine kinase activity from the contractile response of rat aortic rings.

Virtually all studies probing the involvement of tyrosine kinase activity in Ang II activation of vascular smooth muscle have used large arteries or cultured myocytes from these vessels. The involvement of tyrosine kinase activity in evoking Ang II–induced contraction within the microvasculature, including that of the kidney, remains virtually unexplored. Accordingly, the present experiments addressed the hypothesis that tyrosine phosphorylation participates in the renal arteriolar constrictor response to Ang II. Tyrosine kinase activity has also been reported to modulate L-type voltage-gated Ca2+ channels. In particular, tonic phosphorylation by tyrosine kinases has been suggested to maintain the channels in an available state for activation by depolarization.7 These channels are functionally prominent in the afferent arteriole and represent a critical component of the constrictor response to Ang II in this vascular segment.8–11 Accordingly, we also assessed the influence of tyrosine kinase activity on the afferent arteriolar contractile response to membrane depolarization.

Methods

Animals

The procedures used in this study were approved by the University of Nebraska Medical Center Institutional Animal Care and Use Committee and conducted according to the National Institutes of Health guidelines. Experimental animals were obtained from Harlan Sprague-Dawley (Indianapolis, IN), and all efforts were made to minimize animal suffering. The use of animals was in accordance with the guidelines of the American Physiological Society and the American Society for Pharmacology and Experimental Therapeutics. The study was conducted in accordance with the Animal Welfare Act. All procedures used in this study were approved by the University of Nebraska Medical Center Institutional Animal Care and Use Committee and conducted according to the National Institutes of Health guidelines.
Health Guide for the Care and Use of Laboratory Animals. Thirty-two male Sprague-Dawley rats (SAS:VAF strain) weighing 250 to 300 g were purchased from Charles River Laboratories and provided free access to food and water before study.

In Vitro Blood-Perfused Juxtamedullary Nephron Technique

Arteriolar contractile function was assessed in experiments performed with the in vitro blood-perfused juxtamedullary nephron technique.12 After anesthetization with sodium pentobarbital (50 mg/kg IP), a cannula was inserted into the left carotid artery and enalaprilat was administered (2 mg in 1 mL isotonic saline) to reduce endogenous Ang II formation. The left renal artery and vein were ligated and the right renal artery was cannulated through the inferior mesenteric artery, thereby initiating renal perfusion with Tyrode’s solution containing 52 g/L dialyzed BSA and a mixture of L-amino acids.13 Blood was collected through the carotid cannula before harvesting the kidney. Renal perfusion was maintained throughout the ensuing dissection procedure needed to reveal the tubules, glomeruli, and related vasculature of juxtamedullary nephrons. Tight ligatures were placed around the most distal accessible segments of the large arterial branches that supply the exposed microvasculature. Acute surgical papillectomy was performed to avoid an indirect, tubuloglomerular feedback-dependent influence of Ang II on the vasculature.14 The Tyrode’s perfusate was then replaced with reconstituted blood prepared as described previously.13 Renal arterial perfusion pressure was set at 110 mm Hg and maintained at that level throughout the experiment. The perfusion chamber was warmed, and the tissue surface was bathed continuously with Tyrode’s solution containing 10 g/L BSA at 37°C. The tissue was transilluminated on the fixed stage of a compound microscope equipped with a water-immersion objective (×40). Video images of the microvessels were stored on videotape for later analysis. An afferent or efferent arteriole was selected for study, based on visibility and acceptable blood flow. All protocols assessed arteriolar diameter at a single measurement site under several experimental conditions. Afferent arteriolar diameter was monitored at sites ≥100 μm upstream from the glomerulus, whereas efferent arterioles were studied at sites ≤100 μm from the point of emergence from the glomerulus.

Protocols

Effect of Tyrosine Kinase Blockade on Renal Arteriolar Constrictor Responses to Ang II

After a 15-minute stabilization period, afferent or efferent arteriolar baseline diameter was established during an initial 5-minute control period, after which the effects of exogenous Ang II were assessed. Because Ang II causes similar afferent arteriolar constrictor responses when administered from the bath and lumen of in vitro-perfused juxtamedullary nephrons,15 the present studies used the technically simpler method of administering the peptide (1 and 10 nmol/L; 3-minute treatment periods) through the bathing solution. After a 10-minute recovery period, 100 μmol/L tyrphostin AG18 (broad-spectrum tyrosine kinase blocker) was added to the perfusate bath. Ten minutes later, the Ang II exposure sequence was repeated. In other experiments, AG18 was replaced with either 100 μmol/L AG9 (inactive analog) or 100 μmol/L AG1478 (EGF receptor tyrosine kinase blocker). Previous studies from our laboratory have documented the stability of juxtamedullary afferent arteriolar lumen diameter in tissue not exposed to exogenous vasoactive agents for the duration of these experiments.16,17

Effect of Tyrosine Kinase Blockade on Afferent Arteriolar Responses to Depolarization

After the stabilization period, baseline afferent arteriolar diameter was established during exposure to the normal bathing solution (Tyrode’s solution containing 2.7 mmol/L K+). Subsequently, arteriolar diameter responses to increasing extracellular [K+] were assessed (20, 40, and 55 mmol/L K+ bath; substitution for Na+). After recovery from K+-induced depolarization, 100 μmol/L AG18 was added to the bath (10 minutes) and arteriolar responses to increasing bath [K+] were repeated in the continued presence of AG18.

Data Analysis

Microvessel inside diameters were measured from videotaped images by a digital image-shearing monitor (model 908, IPM) calibrated with a stage micrometer, a system that allows diameter measurements reproducible to within <1 μm. Microvessel diameter was measured at 12-second intervals from a single site along the vessel length. The average diameter during the final minute of each treatment period was used for statistical analysis (ANOVA for repeated measures and Newman-Keuls tests). Probability values <0.05 were considered significant. All data are reported as mean±SEM (n=number of arterioles).

Results

Effect of Tyrosine Kinase Blockade on Renal Arteriolar Constrictor Responses to Ang II

Figure 1A illustrates the impact of tyrosine kinase blockade on afferent arteriolar lumen diameter responses to Ang II. Baseline afferent arteriolar diameter averaged 23.1±2.0 μm (n=5) and decreased by 3.4±1.5 and 12.7±2.1 μm on exposure to 1 and 10 nmol/L Ang II, respectively. Removal of Ang II from the bath allowed restoration of afferent diameter to 24.0±2.9 μm (P<0.05 versus baseline). Although AG18 (100 μmol/L) alone did not significantly alter baseline afferent diameter (23.9±2.6 μm), 1 and 10 nmol/L Ang II decreased lumen diameter by only 1.9±0.7 and 8.2±1.5 μm (P<0.05 versus untreated), respectively, during AG18 treatment. Thus, exposure to the broad-spectrum tyrosine kinase blocker reduced afferent arteriolar responsiveness to 10 nmol/L Ang II by 34±9%. However, the time required to develop 50% of the peak response to 10 nmol/L Ang II (t½) was unaffected by AG18, averaging 39±7 seconds before and 47±4 seconds during AG18 treatment (P=0.32 by paired t test).

AG18 also attenuated efferent arteriolar Ang II responsiveness (Figure 1B). Efferent arteriolar lumen diameter averaged 23.6±1.1 μm (n=6) during the untreated baseline period. Ang II evoked reductions in efferent diameter, with 1 nmol/L Ang II decreasing the diameter by 0.7±0.2 μm and 10
nmol/L Ang II decreasing the diameter by 5.0±0.9 μm. Recovery from the Ang II exposure restored efferent diameter to 22.5±1.1 μm (P<0.05 versus baseline). AG18 did not significantly alter efferent arteriolar diameter (22.4±1.1 μm); however, responses to Ang II were attenuated such that 10 nmol/L Ang II only reduced efferent diameter by 2.6±0.6 μm (P<0.005 versus untreated). Thus, tyrosine kinase blockade suppressed the efferent constrictor response to 10 nmol/L Ang II by 52±10%. Values for t½ averaged 38±8 seconds before and 43±17 seconds during AG18 treatment in efferent arterioles (P=0.82 by paired t test), indicating no change in the time course of the response to 10 nmol/L Ang II.

Figure 2A depicts the impact of EGF receptor tyrosine kinase blockade on Ang II–induced afferent arteriolar constriction. Afferent lumen diameter averaged 22.7±1.8 μm (n=4) under baseline conditions and decreased by 5.0±0.8 μm (P<0.05 versus untreated) during exposure to 10 nmol/L Ang II (22.4±1.1 μm). However, responses to Ang II were attenuated such that 10 nmol/L Ang II only reduced afferent diameter by 0.8±0.3 μm (P<0.05 versus untreated). Thus, AG18 treatment in efferent arterioles (2.7 mmol/L K+; 55 mmol/L K+) did not alter baseline afferent arteriolar diameter (22.5±1.2 μm) but suppressed Ang II–induced vasoconstriction. During exposure to 2.7 mmol/L K+ and 55 mmol/L K+ (P<0.001 versus untreated), respectively. Thus, blockade of the EGF receptor tyrosine kinase inhibited the afferent arteriolar response to 10 nmol/L Ang II by 52±8%.

The effect of EGF receptor blockade on afferent arteriolar responsiveness to Ang II is summarized in Figure 2B. Afferent arteriolar diameter averaged 22.5±2.1 μm (n=5) under untreated baseline conditions (2.7 mmol/L K+ and 55 mmol/L K+) and was significantly reduced by 40 mmol/L K+ (Δ=−10.1±3.3 μm) and 55 mmol/L K+ (Δ=−15.5±2.9 μm). Restoration of bath [K+] to 2.7 mmol/L allowed full recovery of afferent diameter to baseline values (22.5±2.1 μm). AG18 did not significantly alter baseline diameter (22.5±2.1 μm) or responses to K+-induced depolarization (40 mmol/L K+, Δ=−10.4±3.2 μm; 55 mmol/L K+, Δ=−14.2±2.2 μm).

**Discussion**

In both afferent and efferent arterioles, Ang II induces constriction through its interaction with AT1 receptors18; however, disparate intracellular signaling events are used by these vascular segments to generate the increase in intracellular Ca2+ concentration necessary to produce vasoconstriction. Available evidence indicates that activation of AT1 receptors in the afferent arteriole activates PLC to elicit IP3-dependent Ca2+ mobilization, resulting in a rise in intracellular Ca2+ concentration.

![Figure 2A](image1.png) ![Figure 2B](image2.png)
cellular [Ca\(^{2+}\)] that increases the open probability of Ca\(^{2+}\)-activated Cl\(^{-}\) channels, with the subsequent membrane depolarization prompting Ca\(^{2+}\) influx through voltage-gated channels.\(^{8,19,20}\) In contrast, activation of efferent arteriolar AT\(_1\) receptors appears to trigger activation of PLC to provoke Ca\(^{2+}\) mobilization and protein kinase C–dependent Ca\(^{2+}\) influx through a voltage-independent mechanism, possibly through store-operated Ca\(^{2+}\) channels.\(^{11,19,20}\) The present study used a pharmacological approach to implicate tyrosine phosphorylation in the Ang II–induced signaling events of both afferent and efferent arterioles.

The role of tyrosine phosphorylation in Ang II–induced constriction was assessed through the use of synthetic tyrphostin compounds with characteristic effects on tyrosine kinase activity. AG18, also known as tyrphostin A23, is a broad-spectrum tyrosine kinase inhibitor that blocks EGF receptor autophosphorylation and platelet-derived growth factor receptor kinase activity only at much higher concentrations (IC\(_{50}\) > 100 \(\mu\)mol/L).\(^{24}\) At concentrations within the range of 100 to 250 nmol/L, AG18 markedly abates control (IC\(_{50}\)) values in the range of 25 to 40 nmol/L.\(^{21,22}\) Because AG18 inhibition of EGF receptor protein kinase is maximally effective at a concentration of 100 \(\mu\)mol/L,\(^{23}\) this concentration was used in the present study. AG1478 is a potent and specific EGF receptor tyrosine kinase inhibitor (IC\(_{50}\) = 3 nmol/L), affecting platelet-derived growth factor receptor kinase activity only at much higher concentrations (IC\(_{50}\) > 100 \(\mu\)mol/L).\(^{24}\) At concentrations within the range of 100 to 250 nmol/L, AG1478 markedly abates EGF-stimulated and Ang II–stimulated increases in MAP kinase activity in rat aortic smooth muscle.\(^{4}\) AG9, also known as tyrphostin A1, is an inactive compound used as a negative control (IC\(_{50}\) > 1250 \(\mu\)mol/L) for EGF receptor kinase.\(^{25}\)

None of the tyrphostin compounds used in this study significantly altered baseline diameter of afferent or efferent arterioles; however, AG18 attenuated both afferent and efferent arteriolar constrictor responses to Ang II. The failure of the inactive tyrphostin analog to alter afferent arteriolar Ang II responsiveness is consistent with the contention that AG18 suppressed Ang II responsiveness through its ability to inhibit tyrosine kinase activity rather than through a nonspecific effect of tyrphostin compounds. These observations are in accord with reports that tyrosine kinase blockade attenuates Ang II–induced pH\(_{\text{in}}\), intracellular [Ca\(^{2+}\)], and/or contractile responses in vascular smooth muscle from aorta or mesenteric artery.\(^{26,28}\)

Tyrphostin compounds have been reported to inhibit L-type voltage-gated Ca\(^{2+}\) channels, either secondary to tyrosine kinase blockade\(^{7,29}\) or through a direct nonspecific (tyrosine kinase–independent) effect on channel activity.\(^{30}\) Because these channels are prominent in the renal afferent arteriole,\(^{9,10}\) and in evoking the vasoconstrictor response of this vessel to Ang II,\(^{8}\) we assessed the impact of AG18 on K\(^{+}\)–induced vasoconstriction. Increases in extracellular [K\(^{+}\)] contract vascular smooth muscle through the effect of membrane depolarization to increase the open probability of L-type Ca\(^{2+}\) channels. We have shown previously that the afferent arteriolar intracellular [Ca\(^{2+}\)] and constrictor responses to this maneuver are blocked by nifedipine and diltiazem.\(^{9,31}\) In contrast with the behavior of nonrenal vascular beds,\(^{6,32–34}\) tyrosine kinase blockade did not alter K\(^{+}\)–induced afferent arteriolar constriction in the present study. This observation has several mechanistic implications. First, the effect of AG18 on Ang II responsiveness cannot be attributed to a direct (tyrosine kinase–independent) effect of AG18 on the L-type Ca\(^{2+}\) channel. Second, it is also unlikely that a direct effect of AG18 on the contractile apparatus or on the Ca\(^{2+}\) sensitivity of the contractile proteins\(^{35,36}\) underlies the impact of this compound on Ang II responsiveness. Finally, it unlikely that either membrane depolarization or the resulting Ca\(^{2+}\) influx initiates the tyrosine kinase activation involved in the afferent arteriolar Ang II signaling. However, we cannot rule out the possibility that the relatively large transient increase in intracellular [Ca\(^{2+}\)] resulting from Ang II–induced Ca\(^{2+}\) mobilization triggers tyrosine kinase activation.

In recent years, it has become clear that activation of various G-protein–coupled receptors (such as the AT\(_1\) receptor) rapidly induces transactivation of the EGF receptor and that inhibition of EGF receptor tyrosine kinase activity prevents the subsequent events that lead to MAP kinase activation and transmission of mitogenic signals to the nucleus.\(^{37}\) AT\(_1\)-EGF receptor cross-talk has been studied primarily in the context of the mitogenic effect of Ang II on aortic myocytes. Although the present study does not address the sequence of events linking AT\(_1\) receptor activation and tyrosine kinase activity to evoke renal arteriolar constriction, involvement of the EGF receptor tyrosine kinase is indicated by the ability of AG1478 to attenuate renal arteriolar constrictor responses to Ang II. These observations provide evidence of cross-talk between the AT\(_1\) receptor and the EGF receptor in both afferent and efferent arterioles, despite differences in other aspects of Ang II–induced signaling at these sites. For example, although Ang II has been suggested to constrict afferent arterioles through G\(_i\) and efferent arterioles through G\(_o\), both of these G proteins are capable of using Src to phosphorylate the EGF receptor for recruitment of adaptors proteins and MAP kinase activation.\(^{38}\) Some studies that used cultured rat vascular smooth muscle suggest that Src family tyrosine kinases mediate Ang II–induced phosphorylation of PLC–\(\gamma\), resulting in IP\(_3\) formation and Ca\(^{2+}\) mobilization.\(^{2,39}\) Alternatively, Ang II may trigger G\(_i\)-mediated PLC–\(\beta\) activation, with the resulting Ca\(^{2+}\) mobilization triggering EGF receptor transactivation and phosphorylation by Src.\(^{4,40}\) The involvement of PLC–\(\beta\) or PLC–\(\gamma\) may represent a critical distinction between these two scenarios; however, although PLC is involved in Ang II–induced signaling in both afferent and efferent arterioles, the role of specific PLC isoforms has not been evaluated. Hence, further investigation is necessary to elucidate the mechanism(s) linking the AT\(_1\) receptor to tyrosine kinase activation in the afferent and efferent arterioles as well as the mechanism through which EGF receptor activation influences contractile tone.

**Summary**

A broad-spectrum tyrosine kinase inhibitor attenuated Ang II responsiveness in both afferent and efferent arterioles but did not significantly alter afferent arteriolar constrictor responses to KCl-induced depolarization. Afferent and efferent arteriolar constrictor responses to Ang II were also diminished by an EGF receptor tyrosine kinase inhibitor. These observations
suggest that tyrosine phosphorylation event(s) contribute to Ang II–induced vasconstrictor signaling in the renal microvasculature and that this process involves the EGF receptor tyrosine kinase. Further studies are required to determine if tyrosine phosphorylation is a critical step mediating Ang II–induced renal arteriolar constriction or, alternatively, if tyrosine kinase activity exerts a modulatory influence on Ang II responsiveness.

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References


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