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Angiotensin II Constriction of Rat Vasa Recta Is Partially Thromboxane Dependent

Erik P. Silldorff, Layla R. Hilbun, Thomas L. Pallone

Abstract—We tested the hypothesis that thromboxane generation mediates vasoconstriction of isolated outer medullary descending vasa recta (OMDVR) by angiotensin (Ang) II. The lipoxygenase and cyclooxygenase (COX) inhibitor eicosatetraynoic acid (1 μmol/L) and the COX inhibitor indomethacin (1 μmol/L) partially reversed Ang II (1 nmol/L) constriction of in vitro perfused OMDVR. To determine whether thromboxane is a mediator of Ang II–induced vasoconstriction, a thromboxane synthase inhibitor, U63577A (1 μmol/L), and thromboxane receptor antagonists, SQ-29548 or BMS-180,291 (1 μmol/L, each), were introduced into the bath of vessels that had been preconstricted by Ang II (1 nmol/L). These agents significantly inhibited vasoconstriction induced by Ang II. In contrast, SQ-29548 and U63577A did not affect vessels preconstricted by raising extracellular KCl from 5 to 100 mmol/L. The thromboxane receptor agonist U46619 (1 μmol/L) constricted OMDVR, an effect that was blocked by the antagonist BMS-180,291. In separate protocols, microperfused OMDVR were pretreated with U63577A or SQ-29548, after which they were exposed to luminal Ang II to induce vasoconstriction. Both agents inhibited vasoconstriction whether preexposure to them was via the bath or the perfusate. We conclude that Ang II–induced constriction of OMDVR is partly mediated by metabolites of arachidonic acid, including thromboxanes. (Hypertension. 2002;40:541-546.)

Key Words: angiotensin II □ thromboxane □ cyclooxygenase □ microcirculation □ renal

The renal microcirculation is arranged in a manner that facilitates separation of blood flow to the cortex, outer medulla, and inner medulla. Descending vasa recta deliver the entire blood supply to the medulla. The existence of contractile pericytes on these vessels suggests a role for them to regulate regional perfusion, a hypothesis that is supported by in vitro evidence demonstrating responsiveness to many vasoactive agents, including angiotensin (Ang) II.1

Ang II type 1 (AT₁) receptor stimulation elicits release of free arachidonic acid into the cytoplasm of tubular epithelial cells,2 endothelium,3 and smooth muscle4 via the stimulation of phospholipase A₂. Arachidonic acid is a substrate for an array of cytosolic enzymes that generate vasoactive metabolites. Ang II has been found to increase the production of prostaglandins in the kidney,5,6 and these agents attenuate the vasoconstrictor response to Ang II.7 The vasodilatory agents include PGE₂ and PGI₂, which are released by glomeruli to attenuate efferent arteriolar constriction.8 Thromboxane A₂ (TxA₂) is a vasoconstrictor eicosanoid that is synthesized within the kidney in response to Ang II.9-12 A role for thromboxanes to mediate Ang II–induced intrarenal vasoconstriction has been established through various observations. In the isolated perfused rabbit kidney, Ang II–induced increase in vascular resistance is blocked by TxA₂ synthase inhibition,10 and Ang II infusion in rats results in increased urinary excretion of TxB₂, the stable metabolite of TxA₂.11,12 Finally, TxA₂/PG_H₉₂ receptor blockade lowers blood pressure in Ang II–dependent hypertension 13.

The medulla receives only a small fraction of total renal blood flow. Therefore, regional effects of thromboxane receptor blockade within the medulla cannot be inferred from measurements of overall renal vascular resistance. In view of this, we tested the hypothesis that cyclooxygenase (COX) products of arachidonic acid mediate vasoconstriction of isolated outer medullary descending vasa recta (OMDVR) by Ang II. Mediation of Ang II–induced constriction by thromboxanes was verified by examining the ability of TxA₂ receptor blockers and a thromboxane synthase inhibitor to reverse or prevent Ang II–induced constriction of isolated perfused OMDVR.

Methods

In Vitro Microperfusion

Young female Sprague-Dawley rats (Harlan, Indianapolis, Ind) were anesthetized by intraperitoneal injection of thiopental (50 mg/kg), after which the kidneys were harvested, sliced, and placed into cold (4°C) buffer (in mmol/L): HEPES 5, NaCl 140, sodium acetate 10, KCl 5, MgCl₂ 1.2, Na₂HPO 4 1.71, NaH₂HPO 4 0.29, CaCl₂ 1, alanine 5, and glucose 5, as well as albumin 0.5 g/dL (pH 7.4). All procedures were performed in accordance with institutional and National Institutes of Health guidelines.

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OMDVR were harvested by microdissection, transferred to the stage of an inverted microscope (Nikon TE-300), cannulated, and perfused at 37°C. The OMDVR perfusate was identical to the HEPES buffer used for microdissection and tissue storage, as defined above. The bath was identical to the perfusate except for addition of hormones and antagonists (Table). The exchange of bath solutions during experiments was controlled by gravity-fed inflow from individual chambers. Perfusion chambers were custom made in our laboratory. Temperature of the perfusion chamber was maintained at 37°C with a feedback system using a CN9111A controller (Omega Engineering). All experiments were preceded by a 20-minute equilibration period.

**Videomicroscopy and Measurement of Vessel Diameters**

To evaluate the effects of vasoactive agents on OMDVR diameters, microperfusion experiments were recorded on videotape using a Panasonic model AG 1980 VCR with a microphone for audio recording of experimental events. The inverted microscope was equipped with a 20%/80% beam splitter and a side port for attachment of a video camera (Dage-MTI, CCD model 72). OMDVR were observed with a 40x objective to yield a final magnification of 1300x on the video screen. During video playback, vessel diameters were measured with calipers at the point of greatest constriction or dilation. Changes in vessel diameter are expressed as percent constriction, defined in terms of the basal diameter in the absence of hormones (Do) and the experimental diameter (D) by the following expression: % constriction = [(1 – D/Do) * 100].

**Experimental Protocols**

In an initial series, we evaluated the ability of a thromboxane synthase inhibitor (U63557A, 1 μmol/L) and thromboxane receptor blockers (SQ-29548, BMS-180,291; each 1 μmol/L) to vasodilate Ang II–preconstricted OMDVR compared with vehicle-treated groups (0.1% dimethyl sulfoxide for SQ-29548). After the equilibration period, OMDVR were exposed to abluminal Ang II (1 nmol/L) and observed for 15 minutes. Vessel diameters were measured every minute. To test the effect of these agents, separate groups were exposed to abluminal inhibitors from minutes 5 to 10 by adding them to the bath. This was followed by a 5-minute washout period. To determine whether the thromboxane analog U46619 could constrict OMDVR, vessels were exposed to U46619 (1 μmol/L) in the presence and absence of the TXA2 receptor antagonist BMS-180,291 (1 μmol/L). Finally, to show specificity for Ang II–induced constriction, the ability of the receptor antagonist SQ-29548 and thromboxane synthase inhibitor U63557A to affect OMDVR constriction by KCl (100 mmol/L, isosmotic substitution for NaCl) was measured in similar protocols.

In a separate series of experiments, the ability of SQ-29548 and U63557A (each 1 μmol/L) to prevent Ang II–induced constriction was examined when vessels were pretreated with these agents from the bath and lumen. In a first protocol, the blockers, or 0.1% dimethyl sulfoxide as control, were added to the bath, after which Ang II (1 nmol/L) was also included to induce vasoconstriction. After 15 minutes of observation during Ang II exposure, the blockers were removed during a 5-minute washout, after which they were reapplied to assess the reversibility of their action. In a second protocol, the same blockers were continuously present in the OMDVR perfusate. After baseline recording, Ang II (1 nmol/L) was added to the bath to induce constriction. After 15 minutes, the blockers were also added to the bath to measure any additional effect of abluminal application.

**Reagents Used to Test Ang II–Thromboxane Interaction**

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Action</th>
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<tbody>
<tr>
<td>ETYA</td>
<td>Lipoxigenase/cyclooxygenase inhibitor</td>
</tr>
<tr>
<td>Indomethacin</td>
<td>Cyclooxygenase inhibitor</td>
</tr>
<tr>
<td>U63557A</td>
<td>Thromboxane synthase inhibitor</td>
</tr>
<tr>
<td>SQ-29548</td>
<td>Thromboxane receptor antagonist</td>
</tr>
<tr>
<td>BMS-180,291</td>
<td>Thromboxane receptor antagonist</td>
</tr>
<tr>
<td>U46619</td>
<td>Thromboxane receptor agonist</td>
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</tbody>
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**Figure 1.** Reversal of Ang II–induced constriction of OMDVR by indomethacin and ETYA. A, Vessels were preconstricted with Ang II (1 nmol/L), after which indomethacin (1 μmol/L, n=12) or vehicle (n=16) was added from 5 to 10 minutes and then washed out (10 to 15 minutes). B, Vessels were preconstricted with Ang II (1 nmol/L), after which ETYA (1 μmol/L, n=7) or 0.1% dimethyl sulfoxide (n=6) was added from 5 to 10 minutes and then washed out (10 to 15 minutes). *P<0.05 vs vehicle-treated group.

**Statistical Analysis**

Experimental results are reported as mean±SE. Statistical comparisons were made using repeated-measures ANOVA or unpaired t test as appropriate. For ANOVA, significance was determined by the Student-Newman-Keuls test. Significance levels were set at P<0.05.

**Results**

**Effect of COX and Lipoxygenase Inhibition on Ang II–Induced Vasoconstriction**

Indomethacin, a COX inhibitor (1 μmol/L, n=12) (Figure 1A), or ETYA, a COX/lipoxygenase inhibitor (1 μmol/L, n=7) (Figure 1B), was added to the bath of OMDVR that had been preconstricted with Ang II. Both agents partially reversed vasoconstriction compared with controls. The vehicle for the control groups in Figure 1A and 1B were water and dimethyl sulfoxide, respectively, at 1:1000 dilution. One vessel in the control series comprising Figure 1A failed to show any significant foci of constriction and was eliminated from the analysis. On removal of either antagonist (indomethacin or ETYA) from the bath, significant recovery of constriction occurred. Notably, the intensification of constriction during washout of indomethacin or ETYA occurred even when time controls exhibited waning of constriction. Indomethacin, ETYA (1 μmol/L), and 0.1% dimethyl sulfoxide did not have an effect on the diameters of OMDVR that had not been preconstricted with Ang II (n=6, 6, and 4, respectively; data not shown).
Effect of Thromboxane Synthase Inhibition and Thromboxane Receptor Antagonism on Ang II–Induced Vasoconstriction

The thromboxane synthase inhibitor U63557A (1 μmol/L, n=7) inhibited Ang II–induced OMDVR constriction (Figure 2), an effect that reversed after it was removed from the bath. Similarly, addition of either of the TxA2 receptor antagonists, SQ-29548 (n=15) (Figure 3A) or BMS-180,291 (n=13) (Figure 3B), inhibited Ang II–induced vasoconstriction. Recovery of constriction also occurred when these agents were removed from the bath. The degree of reversal of inhibition achieved significance for BMS-180,291 but not for SQ-29548. Neither U63557A nor SQ-29548 changed the diameters of OMDVR that had not been preconstricted by exposure to Ang II (n=6 and 7, respectively; data not shown).

Constriction by the Thromboxane Analogue U46619

Abluminal administration of the thromboxane analogue U46619 (1 μmol/L, n=11) induced significant constriction of OMDVR, but the constriction was less intense than that achieved by Ang II (Figure 4; compare with control groups).

Effect of U63557A and SQ-29548 on KCl-Induced OMDVR Vasconstriction

To test the specificity of thromboxane inhibition for blockade of Ang II–induced vasomotion, we determined whether the thromboxane synthase inhibitor U63557A or receptor blocker SQ-29548 (n=6, 1 μmol/L, each group) could inhibit non-specific OMDVR vasconstriction. To depolarize the pericytes and constrict OMDVR, KCl concentration in the bath was raised from 5 to 100 mmol/L by isosmotic substitution for NaCl. KCl induced moderate but stable vasoconstriction that, unlike constriction by Ang II (Figures 2 and 3) was not affected by abluminal exposure to U63557A or SQ-29548 (Figure 5).

Effect of U63557A or SQ-29548 Pretreatment on Ang II–Induced OMDVR Vasconstriction

As shown in Figures 1 through 3, vasoconstriction by Ang II typically wanes after 5 to 10 minutes of abluminal application making interpretation of washout effects difficult (Figures 1 through 3). For added rigor, additional protocols were exe-
cuted in which the synthase inhibitor U63557A, thromboxane receptor blocker SQ-29548, or 0.1% dimethyl sulfoxide (vehicle) was added to the bath 5 minutes before and then during exposure of vessels to Ang II (Figure 6). U6357A, SQ-29548, and dimethyl sulfoxide did not have any effect on OMDVR luminal diameter before Ang II application (data not shown). Vessels pretreated with abluminal SQ-29548 (n=7) constricted significantly less than dimethyl sulfoxide controls (n=5). U6357A and dimethyl sulfoxide controls for SQ-29548, significance was achieved for all times >1 minute. After baseline measurement of luminal diameter, Ang II (1 nmol/L) was added to the bath at 1 minute to induce vasoconstriction. After 15 minutes, the blocker was also added to the bath (15 to 20 minutes) and then washed out. \( *P<0.05 \) vs dimethyl sulfoxide controls. For both SQ-29548 and U63557A, significance was achieved for all times >2 minutes.

**Figure 6.** Effect of abluminal pretreatment with SQ-29548 or U63557A on OMDVR constriction by Ang II. Vessels were pretreated for 5 minutes before time=0 on the abscissa with the thromboxane synthase inhibitor U63557A (1 \( \mu \)mol/L, n=7), the thromboxane synthase inhibitor U63557A (1 \( \mu \)mol/L, n=6), or 0.1% dimethyl sulfoxide (control, n=5) added to the bath, after which Ang II (1 nmol/L) was also added to induce vasosconstriction. The blocker or dimethyl sulfoxide was washed out (15 to 20 minutes) and then reapplied (20 to 25 minutes).

**Figure 7.** Effect of luminal pretreatment with SQ-29548 or U63557A on OMDVR constriction by Ang II. Vessels were pretreated from the time of cannulation with the thromboxane receptor antagonist SQ-29548 (1 \( \mu \)mol/L, n=8), the thromboxane synthase inhibitor U63557A (1 \( \mu \)mol/L, n=5), or 0.1% dimethyl sulfoxide (control, n=6) added to the perfusate. After baseline measurement of luminal diameter, Ang II (1 nmol/L) was added to the bath at 1 minute to induce vasoconstriction. After 15 minutes, the blocker was also added to the bath (15 to 20 minutes) and then washed out. \( *P<0.05 \) vs dimethyl sulfoxide controls. For both SQ-29548 and U63557A, significance was achieved for all times >2 minutes.

**Effect of Luminal Application of U63557A and SQ-29548 on Ang II–Induced OMDVR Vasosconstriction**

In a series of experiments, U63557A (n=5, 1 \( \mu \)mol/L), SQ-29548 (n=8, 1 \( \mu \)mol/L), or 0.1% dimethyl sulfoxide (n=6) was added to the OMDVR perfusate so that vessels were exposed to these agents via the lumen from the time of cannulation and then throughout the experiments. The ability of Ang II (1 \( \mu \)mol/L) to constrict OMDVR was measured for a total of 25 minutes. After the first 15 minutes of Ang II, the blockers or dimethyl sulfoxide were added to the bath to determine whether simultaneous luminal and abluminal exposure would have an additional effect to augment vasodilation. As shown in Figure 7, both agents significantly inhibited Ang II constriction. As previously observed during abluminal application (Figures 3 and 6), SQ-29548 was the more effective blocker. Abluminal exposure from 15 to 20 minutes reversibly augmented vasodilation.

**Discussion**

Arachidonic acid released on activation of phospholipase A\(_2\) (PLA\(_2\)) can be metabolized via the lipoxygenase, COX, and cytochrome P\(_{450}\) pathways to yield vasoactive metabolites that either constrict or vasodilate microvessels. Thus, activation of PLA\(_2\) by Ang II could stimulate vascular smooth muscle to produce vasoconstrictors while simultaneously activating nearby cells to synthesize vasodilators that abrogate vasosconstriction. This is particularly true in the renal medulla, where PGE\(_2\) and PGI\(_2\) can be generated from transporting epithelia, endothelia, and renomedullary interstitial cells.\(^1\) As a consequence of the potential to stimulate synthesis of opposing constrictors and dilators, efforts to dissect pathways of arachidonic acid metabolism with pharmacological inhibitors can lead to inconclusive results. Furthermore, it is possible that differing results will be obtained when pathways of arachidonic acid metabolism are blocked in isolated vessels where cell-to-cell interactions have been eliminated versus whole-organ preparations in which they are intact. Such disparate effects have been obtained in studies of the renal microcirculation.

Early efforts pointed to a role for thromboxanes to mediate intrarenal vasoconstriction by Ang II. Kaushal and Wilson demonstrated that thromboxane synthase inhibition with furagrelate (U63557A) reduced Ang II–induced vasosconstriction.\(^14\) The investigators speculated that this resulted from redirection of arachidonic acid away from thromboxane synthesis to augment substrate for other pathways that form vasodilators. Intravenous infusion of Ang II increased both urinary excretion of the thromboxane metabolite TxB\(_2\) and renal vascular resistance. Wilcox and colleagues\(^11,12\) demonstrated that both thromboxane synthase inhibition and thromboxane receptor antagonism could eliminate the majority of the increase in renal vascular resistance generated by Ang II in the rat. These results were confirmed by others.\(^10–15\)
and a role for thromboxane generation was established in Ang II–dependent 2-kidney, 1-clip Goldblatt hypertension.13–16 More recent experiments in the isolated perfused kidney showed that intrarenal vasoconstriction by endothelin 1 is sensitive to COX and cytochrome P450 blockade, whereas >80% of Ang II–induced vasoconstriction occurs through synthesis of products of the lipoxygenase and COX pathways.17

Single vessel studies have also yielded insights into the ability of arachidonic acid products to influence vasmotion in renal microvessels. In isolated afferent arterioles and OMDVR, exogenous abluminal PGE2 and PGH2 can blunt vasoconstriction by Ang II.18,19 In isolated perfused rabbit afferent and efferent arterioles, Ang II reactivity was enhanced by nonspecific COX blockade with indomethacin.8 In contrast, in the juxtamedullary nephron preparation, indomethacin, NS-398 (COX-2 inhibitor), and piroxicam failed to augment Ang II–induced vasoconstriction of the afferent arteriole.20,21 In our hands, inhibition of COX (indomethacin) (Figure 1A) or combined inhibition of lipoxygenase and COX (ETYA) (Figure 1B) significantly inhibited Ang II constriction suggesting a role for a metabolite of these pathways to mediate signaling by Ang II in OMDVR smooth muscle/pericytes. Our results contrast with the failure of indomethacin to influence Ang II–induced vasoconstriction in the juxtamedullary nephron preparation and might therefore be interpreted to indicate regional differences between cortical arterioles and descending vasa recta. An alternate explanation is that Ang II stimulation of PLA2 in other cell types might favor synthesis of vasodilatory arachidonic acid metabolites from epithelial or interstitial cells that are present in the juxtamedullary nephron preparation.

The medulla of the kidney, receives only a small fraction of total renal blood flow.1 As such, effects on medullary perfusion cannot be inferred from changes in renal vascular resistance. Furthermore, renal microvascular responses can be heterogeneous such that signaling pathways present in cortical vessels might not be reproduced in descending vasa recta.1,22,23 In view of this and the variation of the effect of COX blockade reported in prior studies, we performed experiments to determine whether Ang II constriction of OMDVR is specifically related to thromboxane synthesis in isolated vessels. When either thromboxane synthase (Figure 2) or the thromboxane receptor (Figure 3) was blocked, Ang II constriction of OMDVR was substantially inhibited, supporting a role for TxA2 to mediate a significant component of Ang II–induced OMDVR vasoconstriction. Finally, a synthetic analog of TxA2 (U46619) constricted OMDVR (Figure 4). Both Ang II and U46619 induced vasoconstriction were inhibited by thromboxane receptor blockade with BM-180,291 (Figures 3 and 4), suggesting that a common receptor mediates the response to both agents. Based on these results, we conclude that thromboxanes are a predominant mediator of OMDVR constriction by Ang II.

An additional finding of these studies is that luminal application of a blocker of thromboxane synthesis (U63557A) or a thromboxane receptor antagonist (SQ-29548) inhibits OMDVR constriction by Ang II (Figure 7). Although it is inviting to conclude that this implies release of thromboxanes by endothelial cells, a more conservative interpretation may be in order. Measurements of transport properties of isolated OMDVR have revealed significant permeability to molecules as large as inulin.24 Thus, even when pharmacological agents are placed in the perfusate and not the bath, it can be anticipated that both smooth muscle/pericytes and endothelia will be exposed to them (eg, SQ-29548 and U63557A) (Figure 7). Thus, luminal perfusion cannot lead to firm conclusions concerning the cellular site of origin of vasoactive mediators.

Finally, a note concerning vasoconstriction by KCl (Figure 5) is in order. Raising extracellular KCl from 5 to 100 mmol/L will depolarize the cells that comprise the OMDVR wall.25 In the absence of smooth muscle calcium entry pathways gated by depolarization (eg, L-type and T-type channels), such a maneuver might not be expected to yield vasoconstriction. In contrast to this, stable moderate vasoconstriction was observed on KCl depolarization (Figure 5) a finding that favors the existence of voltage gated calcium channels in OMDVR pericytes. This supports the recent data of Hansen et al26 who used polymerase chain reaction and immunohistochemistry to identify L-type and T-type subunits in smooth muscle of the juxtamedullary circulation including efferent arterioles and vasa recta.

Perspectives

In summary, hormonal regulation of perfusion of the renal inner and outer medulla via modulation of OMDVR contractility might play a vital role in the physiological processes of urinary concentration and pressure natriuresis.1,27 In addition, the anatomical separation of inner and outer medullary blood flow via descending vasa recta in outer medullary vascular bundles might suggest a role for alteration of descending vasa recta constriction in the mediation of ischemic acute renal failure. The current results show an important role for thromboxanes in constricting OMDVR and may help to explain the recent observation that experimental acute renal failure is accompanied by enhanced vascular reactivity to Ang II through activation of TxA2 receptors.28,29

Acknowledgments

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References


