Endothelium-Derived Nitric Oxide Modulates Vascular Action of Aldosterone in Renal Arteriole

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Abstract—We have recently demonstrated that aldosterone causes nongenomic vasoconstriction by activating phospholipase C (PLC) in the preglomerular afferent arteriole (Af-Art). In the present study, we tested the hypothesis that endothelium modulates this vasoconstrictor action by releasing nitric oxide (NO). In addition, to study the post-PLC mechanism, we examined possible contributions of phosphoinositol hydrolysis products. Rabbit Af-Arts were microperfused at 60 mm Hg in vitro, and increasing doses of aldosterone (10\(^{-10}\) to 10\(^{-5}\) mol/L) were added to the bath and lumen. Aldosterone caused dose-dependent vasoconstriction (within 10 minutes); significant (P<0.01) constriction was observed from 5×10\(^{-9}\) mol/L, and at 10\(^{-8}\) mol/L, intraluminal diameter decreased by 29%±3% (n=9). Disrupting the endothelium augmented vasoconstriction; significant constriction was observed from 10\(^{-10}\) mol/L, and at 10\(^{-8}\) mol/L, the diameter decreased by 38%±2% (n=6). NO synthesis inhibition reproduced this augmentation (n=7). Pretreatment with chelerythrine (10\(^{-8}\) mol/L), a protein kinase C (PKC) inhibitor, slightly attenuated the constriction; aldosterone at 10\(^{-8}\) mol/L now decreased the diameter by 18%±3% (n=7). However, in Af-Arts treated with thapsigargin (10\(^{-6}\) mol/L) or dantrolene (3×10\(^{-5}\) mol/L), which blocks inositol 1,4,5-triphosphate (IP\(_3\)) and diacylglycerol (DAG); IP\(_3\) induces intracellular calcium release, aldosterone at 10\(^{-8}\) mol/L decreased the diameter by only 9%±1% (n=6) or 9%±2% (n=5), respectively. These results demonstrate that in the Af-Art endothelium-derived NO modulates vasoconstrictor actions of aldosterone that are mediated by the activation of both IP\(_3\) and PKC pathways. Such vasoconstrictor actions of aldosterone may contribute to the development or aggravation of hypertension by elevating renal vascular resistance in cardiovascular diseases associated with endothelium dysfunction. (Hypertension. 2004;43[part 2]:352-357.)

Key Words: aldosterone | endothelium | nitric oxide | arterioles | phospholipases | protein kinases | inositol

Recent studies provide evidence that aldosterone (Aldo) accelerates hypertension and glomerulosclerosis in animal models of various renal diseases, including malignant hypertension.\(^1\)-\(^4\) Although the underlying mechanisms for these actions are not well defined, Aldo may exert these deleterious renal effects by elevating renal vascular resistance besides its genomic “volume-retaining” actions. Indeed, we have recently demonstrated that Aldo causes nongenomic vasoconstriction in the preglomerular afferent arteriole (Af-Art),\(^5\) a crucial vascular segment to the control of renal vascular resistance.\(^6\) In that study we found that Aldo causes vasoconstriction by activating phospholipase C (PLC), with a subsequent calcium mobilization through L-type voltage-dependent calcium channels (L-VDCC). However, factors that modulate the vasoconstrictor action of Aldo in the Af-Art have not been defined.

In the present study, we tested the hypothesis that the endothelium modulates vasoconstrictor action of Aldo by releasing vasodilator substances. For this, we microdissected and perfused rabbit Af-Arts in vitro and examined the effects of endothelial disruption and inhibition of the synthesis of endothelium-derived vasodilators on Aldo-induced constriction. In addition, activation of PLC is known to induce hydrolytic breakdown of phosphoinositides into inositol 1,4,5-triphosphate (IP\(_3\)) and diacylglycerol (DAG); IP\(_3\) induces intracellular calcium release from the sarcoplasmic reticulum and DAG activates protein kinase C (PKC).\(^7\) To study the post-PLC mechanism for vascular actions of Aldo, we examined the possible contribution of these phosphoinositol hydrolysis products to Aldo-induced vasoconstriction in Af-Arts.

Methods

Isolation and Microperfusion of the Rabbit Af-Art

This study was performed in accordance with the Guide for Animal Experimentation, Tohoku University School of Medicine. We used methods described previously to microperfuse rabbit Af-Arts.\(^8\) In brief, a single superficial Af-Art with intact glomerulus was microdissected from the kidney of each male New Zealand White rabbit (1.5 to 2.0 kg) and transferred to a temperature-regulated chamber mounted on an inverted microscope (IMT-2; Olympus, Tokyo). The arteriole was cannulated with an array of glass pipettes and perfused with oxygenated medium 199 (GIBCO BRL, Grand Island, NY).

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containing 5% BSA (Sigma, St. Louis, Mo). Intraluminal pressure was maintained at 60 mm Hg throughout the experiments. The bath (medium 199 containing 0.1% BSA) was exchanged continuously. Microdissection and cannulation of the arteriole were completed within 90 minutes at 8°C, after which the bath was gradually warmed to 37°C for the rest of the experiment. Once the temperature was stable, a 30-minute equilibration period was allowed before taking any measurements. Images of the arteriole were recorded with a video system.8,9 When we analyzed the data, we first observed the whole record of each experiment and decided which site had the strongest constriction occurrence in response to Aldo. We consistently measured the intraluminal diameter of this site throughout the experiments (including the control period or pretreatment period).

**Experimental Protocols**

**Protocol 1: Effect of Aldo on the Luminal Diameter**

After the equilibration period, increasing doses of Aldo (10⁻¹⁻⁰, 10⁻⁹, 5×10⁻⁸, and 10⁻⁷ mol/L; Sigma) were added to the bath and lumen. The diameter was measured immediately before adding Aldo and observed for 20 minutes at each dose. We previously demonstrated that nongenomic vasoconstrictor actions of Aldo on Af-Arts reach maximum within 10 minutes, with the diameter remaining at the level for 30 minutes.5

**Protocol 2: Effect of Endothelial Disruption**

We examined the possible modulatory role of the endothelium. As described previously,10,11 we used antibodies against human factor VIII-related antigen and guinea pig complements to selectively disrupt endothelial cells without altering the function of vascular smooth muscle cells. After the endothelial disruption, vasoconstrictor actions of Aldo were examined as in protocol 1.

**Protocol 3: Effect of Inhibiting the Synthesis of Endothelium-Derived Vasodilators**

We examined the possible contribution of endothelium-derived vasodilators by inhibiting the synthesis of NO or prostaglandin. NO synthesis was inhibited with N⁵-nitro-L-arginine methyl ester (L-NAME; Sigma) at 10⁻⁴ mol/L, which blocks acetylcholine, an endothelium-dependent vasodilator, -induced vasodilation in rabbit Af-Arts.5 Cyclooxygenase activity was inhibited with indomethacin (Indo; Sigma) at 5×10⁻⁴ mol/L, which blocks the effect of arachidonic acid (10⁻⁴ mol/L) on renin release in rabbit Af-Arts.12 After the equilibration period, Af-Arts were treated with L-NAME or Indo for 30 minutes. Then, vasoconstrictor actions of Aldo were examined as in protocol 1.

**Protocol 4: Effect of Increasing Basal Tone**

Because L-NAME not only augmented Aldo-induced vasoconstriction but also reduced basal diameter (see Results), we tested whether L-NAME affected the Aldo action by increasing basal tone. The experimental design was the same as that in protocol 3, except that basal diameter was reduced with norepinephrine (Sigma) at 3 to 5×10⁻⁷ mol/L instead of 10⁻⁴ mol/L; L-NAME.

**Protocol 5: Effect of PKC Inhibition**

To study the possible contribution of PKC activation to Aldo-induced vasoconstriction, we examined the effect of chelerythrine, a specific PKC inhibitor.13 On the day of the experiment, a fresh solution containing chelerythrine (Sigma) at 10⁻⁴ mol/L was prepared in physiological salt solution containing 0.1% DMSO (Sigma). After the equilibration period, Af-Arts was treated with chelerythrine at 10⁻⁴ mol/L for 30 minutes. Then, vasoconstrictor actions of Aldo were examined as in protocol 1. This concentration of chelerythrine completely abolished Aldo-vasoconstriction induced by PKC activation without acting on L-VDCC.14

**Protocol 6: Effect of Inhibiting the Intracellular Calcium Mobilization**

We studied the possible contribution of calcium release from sarcoplasmic reticulum to Aldo-induced constriction. For this, we examined the effect of thapsigargin, an sarcoplasmic reticulum calcium pump inhibitor.15 After the equilibration period, Af-Arts were treated with thapsigargin (Sigma) at 10⁻⁶ mol/L for 30 minutes. Then, vasoconstrictor actions of Aldo were examined as in protocol 1. Thapsigargin at this concentration abolishes angiotensin II-induced Af-Art vasoconstriction, which is mainly mediated by IP₃-induced calcium release from intracellular stores,14,16 without acting on L-VDCC in Af-Arts.16,17 We also examined the effect of dantrolene (Sigma), another intracellular calcium release blocker,18 at 3×10⁻⁴ mol/L. Dantrolene at this concentration had similar effect as that of thapsigargin at 10⁻⁷ mol/L in Af-Arts.14 We used the same vehicle for thapsigargin or dantrolene with that for chelerythrine.

**Data Analysis**

Values were expressed as mean±SEM, and all statistical analyses were performed using percent changes. Student paired t test was used to examine whether the diameter at a given concentration differed from the control or pretreated period within each group. For this, Bonferroni multiple comparison adjustment was used to reduce the significance level to 0.0125 (0.05/4; Bonferroni adjustment for 4 doses). ANCOVA was used to examine whether dose–response curves differed between groups, and a 2-sample t test was used to examine whether the change in diameter at a given concentration differed between groups. A value of P<0.05 was considered significant for this analysis.

**Results**

**Protocol 1: Effect of Aldo on the Luminal Diameter**

Basal luminal diameter of Af-Arts was 17.2±0.3 μm (n=9). As previously reported,8 Aldo caused dose–dependent constriction in Af-Arts (Figure 1). Significant constriction was observed from 5×10⁻⁷ mol/L, which decreased the diameter by 3.6±0.6 μm (or 21%±4%; P<0.001); at 10⁻⁸ mol/L, the diameter decreased by 5.0±0.4 μm (or 29±3%). At each dose, the constriction reached maximum within 10 minutes and persisted throughout the observation period.

**Protocol 2: Effect of Endothelial Disruption**

Treatment with antibodies against factor VIII-related antigen and complements did not alter the luminal diameter of Af-Arts, possible mechanisms of which were discussed previously;10 the diameter before and after the treatment was 17.4±0.7 and 17.0±0.8 μm (n=6). As shown in Figure 1, in endothelium-disrupted Af-Arts, Aldo began to cause significant constriction from as low as 10⁻¹⁰ mol/L (by 1.8±0.4 μm or 10%±3%; P<0.01); at 10⁻⁹, 5×10⁻⁹, or 10⁻⁸ mol/L, the diameter decreased by 25%±4%, 34%±2%, or 38%±2%, respectively. Thus, endothelial disruption significantly (P<0.01) augmented vasoconstrictor actions of Aldo at 10⁻⁹ and 5×10⁻⁹ mol/L. At the end of each experiment, we confirmed that Af-Arts did not dilate in response to acetylcholine (10⁻⁵ mol/L; Sigma).

**Protocol 3: Effect of Inhibiting the Synthesis of Endothelium-Derived Vasodilators**

NO synthesis inhibition with L-NAME decreased the diameter by 22%±4% from 17.2±0.6 to 13.4±0.7 μm (n=7), whereas cyclooxygenase inhibition with Indo did not affect basal diameter; the diameter before and after the treatment was 16.9±0.4 and 16.4±0.4 μm (n=6). Pretreatment with L-NAME significantly augmented vasoconstrictor actions of Aldo (Figure 1). In L-NAME-treated Af-Arts, Aldo began to cause significant constriction from 10⁻¹⁰ mol/L (12%±3%;
Protocol 4: Effect of Increasing Basal Tone
Pretreatment with norepinephrine reduced basal diameter by 23%±3%, 29%±2%, or 36%±2%, respectively. Thus, NO synthesis inhibition with L-NAME had similar effects as those of endothelial disruption on the vasoconstrictor action of Aldo; there was no difference in Aldo-induced constriction between endothelium-disrupted and L-NAME-treated Af-Arts. However, Indo had no effect. In Indo-treated Af-Arts, Aldo began to cause significant constriction from 5×10⁻⁹ mol/L, which decreased the diameter by 3.0±0.3 μm (or 18%±2%; P<0.001); at 10⁻⁸ mol/L, the diameter decreased by 4.0±0.5 μm (or 24%±4%).

Protocol 5: Effect of PKC Inhibition
Pretreatment with chelerythrine did not affect the luminal diameter of Af-Arts. The diameter before and after the treatment was 17.3±0.6 and 17.2±0.6 μm (n=7). In chelerythrine-treated Af-Arts, Aldo began to cause significant constriction from 5×10⁻⁹ mol/L, (by 2.7±0.4 μm or 16%±2%; P<0.001); at 10⁻⁸ mol/L, the diameter decreased by 3.1±0.5 μm (or 18±3%). Thus, PKC inhibition with chelerythrine slightly but significantly (P<0.01) attenuated vasoconstrictor actions of Aldo at 10⁻⁸ mol/L (Figure 3, in which non-treated group represents Af-Arts studied in protocol 1). We confirmed that chelerythrine vehicle has no effect on the luminal diameter or vascular reactivity of Af-Arts.

Protocol 6: Effect of Inhibiting the Intracellular Calcium Mobilization
Pretreatment with thapsigargin or dantrolene did not affect the luminal diameter of Af-Arts. The diameter before and after the treatment was 17.2±0.5 and 16.6±0.5 μm for thapsigargin (n=6) or 17.4±0.4 and 16.5±0.8 μm for dantrolene (n=5). Both thapsigargin and dantrolene markedly attenuated the vasoconstriction (Figure 3). Aldo had no effect until the concentration reached 10⁻⁸ mol/L, which decreased the diameter by only 1.5±0.2 μm (or 9±1%) or
1.5±0.4 μm (or 9%±2%) in thapsigargin- or dantrolene-treated Af-Arts, respectively. In addition, when compared with chelerythrine-treated Af-Arts, vasoconstrictor actions of Aldo at 5×10⁻⁹ mol/L were significantly (P<0.005) less in these arterioles.

**Discussion**

We recently demonstrated that Aldo at the nmol/L level causes dose-dependent vasoconstriction in the Af-Art.⁵ The major characteristic of the vasoconstrictor action was its relatively early onset (apparent within 5 minutes and reaches maximum within 10 minutes), which was not affected by spironolactone (a mineralocorticoid receptor antagonist) and was reproduced by membrane-impermeable albumin-conjugated Aldo. Furthermore, neither actinomycin D nor cycloheximide (inhibitors of transcription or protein synthesis) had effect. Taken together, we concluded that the vasoconstrictor action of Aldo on the Af-Art is nongenomic. In the present study, to study the vascular action of Aldo further, we tested the hypothesis that the endothelium modulates this vasoconstriction, because endothelium modulates Af-Art constriction induced by several stimuli (such as angiotensin II and myogenic response).¹¹,¹⁹,²⁰ We found that endothelial disruption increases the sensitivity of Af-Art to Aldo and augments the vasoconstrictor actions. These effects were reproduced by pretreatment with L-NAME but not with Indo, indicating that vasodilator prostaglandin does not modulate vasoconstrictor actions of Aldo, although Aldo exerts nongenomic stimulation of cAMP (which mediates vasodilator actions of prostaglandin)²¹ production in vascular smooth muscle cells and inner medullary collecting ducts.²³ It is unlikely that effects of L-NAME were caused by changes in basal tone, because a similar reduction in basal diameter with norepinephrine had no effect on Aldo-induced vasoconstric-

Thus, we believe that endothelium-derived NO modulates nongenomic vasoconstrictor actions of Aldo in Af-Arts. This notion is confirmed by the finding that L-NAME does not augment Aldo action when administered to endothelium-disrupted Af-Arts (n=3, data not shown).

Our present and previous studies⁵ demonstrate that the concentrations of Aldo required for significant Af-Art constriction were much higher than physiological plasma levels (≈10⁻¹⁰ mol/L in human²⁴ or ≈5×10⁻¹⁰ mol/L in rabbit²⁵). However, the present study suggests that Aldo at physiological concentrations may cause significant vasoconstriction in Af-Arts after the occurrence of endothelial dysfunction, which often associates with hypertension.²⁶ Thus, such vasoconstrictor actions of Aldo on Af-Arts may play an important role in the aggravation of hypertension by elevating renal vascular resistance. Additionally, in other diseases associated with impaired endothelial function such as hyperlipidemia and diabetes mellitus, Aldo may promote a development of hypertension, because the Af-Art accounts for most of the preglomerular vascular resistance and an increase in its vascular resistance contributes to the pathogenesis of essential hypertension.⁶

In addition to nongenomic actions, genomic renal deleterious actions of Aldo have also been reported. Rocha et al.⁵,²⁷ have demonstrated that spironolactone or eplerenone, a selective Aldo receptor blocker, reduces proteinuria and malignant nephrosclerotic lesions without altering blood pressure in hypertensive rats with renal dysfunction. In addition, recent studies provide evidence that Aldo, through its genomic actions, causes endothelial dysfunction by increasing oxidative stress.²⁸,²⁹ Thus, both genomic and nongenomic pathways may synergistically contribute to the vascular actions of Aldo on the Af-Art. It may be possible that endothelial dysfunction induced by genomic actions impairs
buffering effects of NO, which in turn triggers nongenomic vasoconstrictor actions of Aldo on the Af-Art. Further studies examining possible genomic vascular actions of Aldo on the renal hemodynamics, together with their possible interactions with nongenomic actions, especially in vivo, are clearly required.

We have recently demonstrated that Aldo causes nongenomic vasoconstriction in the Af-Art by activating PLC with a subsequent calcium mobilization through L-VDCC,5 however, the precise mechanism of post-PLC activation was unclear. Thus, in the present study, we examined the possible contribution of IP3 and DAG-PKC pathways to Aldo-induced vasoconstriction. We found that inhibition of either the Ca2+-release from IP3-sensitive intracellular calcium stores (with thapsigargin or dantrolene) or the PKC activity (with chelerythrine) significantly attenuates Aldo-induced vasoconstriction. While results were obtained by pharmacological interventions and there are some limitations (ie, specificity of the inhibitors), our results suggest the possibility that IP3 and DAG-PKC pathways act in concert to elicit Aldo-induced vasoconstriction. Our findings are consistent with those of Christ et al30,31 that Aldo, presumably through its nongenomic actions, stimulates the production of IP3 and DAG-PKC in vascular smooth muscle cells. However, because thapsigargin or dantrolene inhibited Aldo-induced vasoconstriction in a much stronger way than chelerythrine did, it is suggested that the IP3+ pathway plays a more important role than the DAG-PKC pathway. This notion may be supported by the findings that Af-Art vasoconstriction induced by PLC activation is mainly mediated by IP3+-induced intracellular calcium mobilization that is required to activate L-VDCC,14,16,17,32 whereas DAG-PKC pathway indirectly modulates this constriction through its cross-talk to the IP3+/L-VDCC pathway.14 However, because these findings were obtained using other vasoconstrictors than Aldo (such as angiotensin II and NE) for PLC activation, possible cross-talk between IP3 and DAG-PKC pathways in the vasoconstrictor mechanisms for Aldo remain to be clarified.

In conclusion, our results demonstrate that endothelium-derived NO modulates vasoconstrictor actions of Aldo on the Af-Art and suggest that by elevating renal vascular resistance, Aldo (even at physiological concentrations) may contribute to the development or aggravation of hypertension in cardiovascular diseases associated with endothelium dysfunction. We also found that both IP3 and DAG-PKC pathways act in concert to elicit Aldo-induced vasoconstriction, although IP3 pathway may play a more important role. However, our results may represent an in vitro phenomenon and may have no direct relevance to the in vivo Aldo action. Thus, to clarify the physiological and pathophysiological significance of the vascular actions of Aldo in the kidney, studies investigating the renal hemodynamic effects of Aldo in vivo are required.

Perspectives

Recent studies suggest the important clinical implications of Aldo in the pathophysiology of cardiovascular and progressive renal diseases. Although it has been initially anticipated that such adverse effects could be mitigated by blocking Aldo synthesis using angiotensin-converting enzyme inhibitors or angiotensin receptor antagonists, this is not the case because Aldo escape (or breakthrough) occurs during long-term administration of angiotensin-converting enzyme inhibitors or angiotensin receptor antagonists.33,34 Therefore, it is now proposed that the use of Aldo-receptor antagonists in addition to angiotensin-converting enzyme inhibitors (or angiotensin receptor antagonists) has additional benefit in the prevention of end-organ damage in cardiovascular and renal diseases.35–37 However, in the present and previous5 studies, we found that Aldo causes nongenomic vasoconstriction in renal arterioles, especially in the presence of endothelium dysfunction. Because clinically available Aldo antagonists at present (spironolactone or eplerenone) cannot inhibit such nongenomic vascular actions of Aldo,5 complete blockade of Aldo action (both genomic and nongenomic actions) would exert superior cardioprotective or renoprotective effects than do these Aldo antagonists. Thus, development of new therapeutic compounds that can inhibit rapid nongenomic Aldo actions, or even the nongenomic and genomic actions, could yield therapeutic benefits in a variety of clinical fields, including cardiovascular and progressive renal diseases.

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