Original Article

Aldosterone Enhances Ligand-Stimulated Nitric Oxide Production in Endothelial Cells

Akiko MUTOH[1)], Masashi ISSHIKI[1)], and Toshiro FUJITA[1)]

Chronic and acute actions of aldosterone have been shown recently to directly affect the cardiovascular system. However, it is unclear whether the acute effects of aldosterone on vasculature are constrictive or dilatory. Here, to clarify the nongenomic effects of aldosterone on endothelial function, we examined the effects of aldosterone on nitric oxide (NO) production in cultured endothelial cells (ECs) and on vascular tone. The intracellular NO production of bovine aortic ECs loaded with DAF-2 was determined using confocal microscopy. Accumulated NO in the culture medium was quantified by a microplate reader using membrane-impermeable DAF-2. Phosphorylation of endothelial NO synthase (eNOS) at Ser^{1179} was assessed by Western blotting. Changes in intracellular Ca^{2+} ($[Ca^{2+}]_i$) were determined by confocal microscopy in ECs doubly loaded with fluo-4 and Fura Red. The effects of aldosterone, acetylcholine (ACh), and other signaling molecules on the tension of phenylephrine (PE)-contracted aortas of Sprague-Dawley rats were examined in an *ex vivo* organ bath chamber system. Short-term pre-exposure to aldosterone (1×10^{-7} mol/L) enhanced ATP-induced NO production in ECs with increased phosphorylation of eNOS at Ser^{1179}. These effects were blocked by eplerenone, a mineralocorticoid receptor (MR) antagonist, and LY294002, a phosphatidylinositol 3-kinase (PI3K) inhibitor. Notably, aldosterone alone did not affect ATP-induced $[Ca^{2+}]_i$ changes or the Ser^{1179} phosphorylation. Similarly, aldosterone (1×10^{-8} to 1×10^{-7} mol/L) did not affect the tone of rat aortas precontracted by PE, but enhanced ACh-induced vasorelaxation, which was again reversed by eplerenone or LY29400. In contrast, sodium nitroprusside–induced vasorelaxation in endothelium-denuded aortas was not affected by aldosterone. Thus, aldosterone acutely enhances ligand-mediated endothelial NO production by eplerenone-sensitive mechanisms involving a PI3K that may synergize Ca^{2+}-dependent eNOS phosphorylation at Ser^{1179}. (*Hypertens Res* 2008; 31: 1811–1820)

***Key Words*: nongenomic action, endothelium, mineralocorticoid receptor, phosphoinositide 3-kinase, eplerenone**

Introduction

Aldosterone has been recognized traditionally as one of the mediators regulating homeostasis of water and sodium through mineralocorticoid receptor (MR)–mediated genomic actions on renal tubular cells. However, experimental evidence has recently indicated that MR expression is not confined to renal tissues and that aldosterone directly affects non-epithelial cardiovascular cells. The Randomized Aldactone Evaluation Study (RALES) (*1*) carried out in 1999 demonstrated highly beneficial effects of an MR antagonist on heart failure. Since then, MR antagonists have also been shown to increase endothelial function (*2*) and improve the prognosis of myocardial infarction with left ventricular dysfunction or heart failure (*3*). These studies suggest a close link between aldosterone and cardiovascular pathophysiology.

Although the classical actions of aldosterone are genomi-

From the [1)]Department of Nephrology and Endocrinology, Graduate School of Medicine, University of Tokyo, Tokyo, Japan.

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Address for Reprints: Masashi Isshiki, M.D., Ph.D, Department of Nephrology and Endocrinology, Graduate School of Medicine, University of Tokyo, 7–3–1 Hongo, Bunkyo-ku, Tokyo 113–8655, Japan. E-mail: isshiki-tky@umin.ac.jp

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cally exerted through MR-mediated transcription, aldosterone also works non-genomically like other steroid hormones (*4*, *5*). Acute actions of aldosterone have been reported in cells derived from various tissues including renal epithelial cells (*6*, *7*), cardiac myocytes (*8*), endothelial cells (ECs) (*9*, *10*) and vascular smooth muscle cells (VSMCs) (*11*). Signaling mechanisms of those nongenomic actions can involve intracellular Ca^{2+} ($[Ca^{2+}]_i$), cAMP, protein kinase C (PKC), phosphatidylinositol 3-kinase (PI3K), Na^+-H^+ transport–linked pH changes, and extracellular signal–regulated kinase (ERK)1/2 depending on the cell type (*12*, *13*). Schneider *et al*. (*10*), Wehling *et al*. (*14*), and Haseroth *et al*. (*15*) observed that aldosterone directly elevates spironolactone-insensitive $[Ca^{2+}]_i$ in ECs (*10*), VSMCs (*14*), and even MR-deficient cells (*15*) and proposed that such action is mediated by a non-classical MR. In contrast, nongenomically elevated $[Ca^{2+}]_i$ by aldosterone in mesenteric arterial cells was reported to be blocked by eplerenone (*16*). Liu *et al*. (*9*) could not detect any $[Ca^{2+}]_i$ changes in VSMCs and found that aldosterone acutely stimulated endothelial NO production that was blocked by an MR antagonist. Studies of the nongenomic actions of aldosterone on vasculature, specifically on rabbit afferent arterioles (*17*, *18*) or human forearm blood flow (*19*, *20*), have led to inconsistent conclusions as to whether aldosterone is constrictive or dilatory

To provide insight into the nongenomic effects of aldosterone on endothelial function and to clarify the previously reported conflicting results, we examined the effects of aldosterone on nitric oxide (NO) production and $[Ca^{2+}]_i$ in cultured ECs as well as on the vascular tone of rat aortic rings.

Methods

Materials

LY294002, L-nitro arginine methyl ester (L-NAME), ATP, dimethylsulfoxide (DMSO), corticosterone, and RU486 were purchased from Sigma-Aldrich Co. (St. Louis, USA). DAF-2 DA and DAF-2 were purchased from Daiichi Pure Chemicals Co. (Tokyo, Japan). Fluo-4 AM and Fura Red AM were purchased from Molecular Probes, Invitrogen Corp. (Carlsbad, USA). Ionomycin was purchased from Calbiochem, EMD Biosciences (La Jolla, USA). Eplerenone was obtained from Pfizer Inc. All other reagents were purchased from Wako Pure Chemical Industries (Osaka, Japan).

Cells

ECs were isolated from bovine fetuses by mechanically scraping the intima of the descending thoracic aorta with a razor and then immediately suspending the sample in M199 (GIBCO, Invitrogen Corp.) supplemented with 20% complement-removed fetal bovine serum (FBS) (JRH Biosciences Inc., Lenexa, USA) and 1% penicillin-streptomycin-glutamine (100×) (GIBCO, Invitrogen Corp.). These preparations were performed in a slaughterhouse. The cell suspensions were kept in a cool box for 3 to 4 h until the mothers of the fetuses were confirmed to be negative for bovine spongiform encephalopathy (BSE). After centrifugation, cells were resuspended in medium and incubated in a humidified CO_2 incubator at 37°C. ECs were passaged before full confluence and used before passage (*15*). To eliminate the effects of steroid hormones in the serum, cells were incubated in M199 supplemented with 20% FBS treated with dextran-charcoal for 3–5 d before the experiment.

Nitric Oxide Imaging

ECs seeded on glass-bottomed culture dishes (MatTek Corporation, Ashland, USA) were incubated with 1×10^{-7} mol/L aldosterone or vehicle for 5 min, washed with phosphate-buffered saline (PBS) and incubated for 20 min in Opti-MEM (GIBCO, Invitrogen Co.) with 1×10^{-5} mol/L DAF-2 DA and 0.05% Pluronic F127 (Molecular Probes, Invitrogen Corp.) at 37°C. Then, the medium was changed to M199 with 20% dextran-charcoal–treated FBS and incubated for another 5 min. Cells were washed with Hanks' balanced salt solution (HBSS; GIBCO, Invitrogen Corp.) containing 141.7 mmol/L Na^+, 1.2 mmol/L Ca^{2+}, 1.05 mmol/L $MgCl_2$, and 0.83 mmol/L $MgSO_4$. For confocal microscopy, cells loaded with DAF-2 were excited at 488 nm and emissions ranging from 500 to 540 nm were obtained using a Leica confocal laser scanning microscope system (Leica Microsystems, Wetzler, Germany). Images were acquired every 10 s by a built-in program in the confocal system. The processing of ratiometric images was done off-line using the ratio imaging module of OpenLab software (ImproVision Inc., Lexington, USA).

Nitric Oxide Assay

ECs were seeded on 96-well microplates. Following pre-incubation of the cells with either an inhibitor (1×10^{-5} mol/L eplerenone, 1×10^{-5} mol/L LY294002, 1×10^{-4} mol/L L-NAME, 1×10^{-5} mol/L RU486) or vehicle for 2 h, either aldosterone (1×10^{-9} – 1×10^{-6} mol/L) or vehicle was added into the medium for 10 min. At this point, DAF-2 (1×10^{-5} mol/L final concentration) was added to the medium, and then either ATP (1×10^{-5} mol/L final concentration) or vehicle was applied for 1 h. Fluorescence of the supernatant was measured by a microplate reader (ARVO MX/Light; PerkinElmer, Waltham, USA) using a 485 nm excitation filter and a 535 nm emission filter.

Ca^{2+} Imaging

For $[Ca^{2+}]_i$ imaging, fluo-4 (1×10^{-5} mol/L) and Fura Red (1×10^{-5} mol/L) were loaded into cells grown on glass-bottomed culture dishes with the same technique used for DAF-2 loading. Cells in HBSS were excited at 488 nm and emissions ranging from 500–540 nm (fluo-4) and 600–680 nm

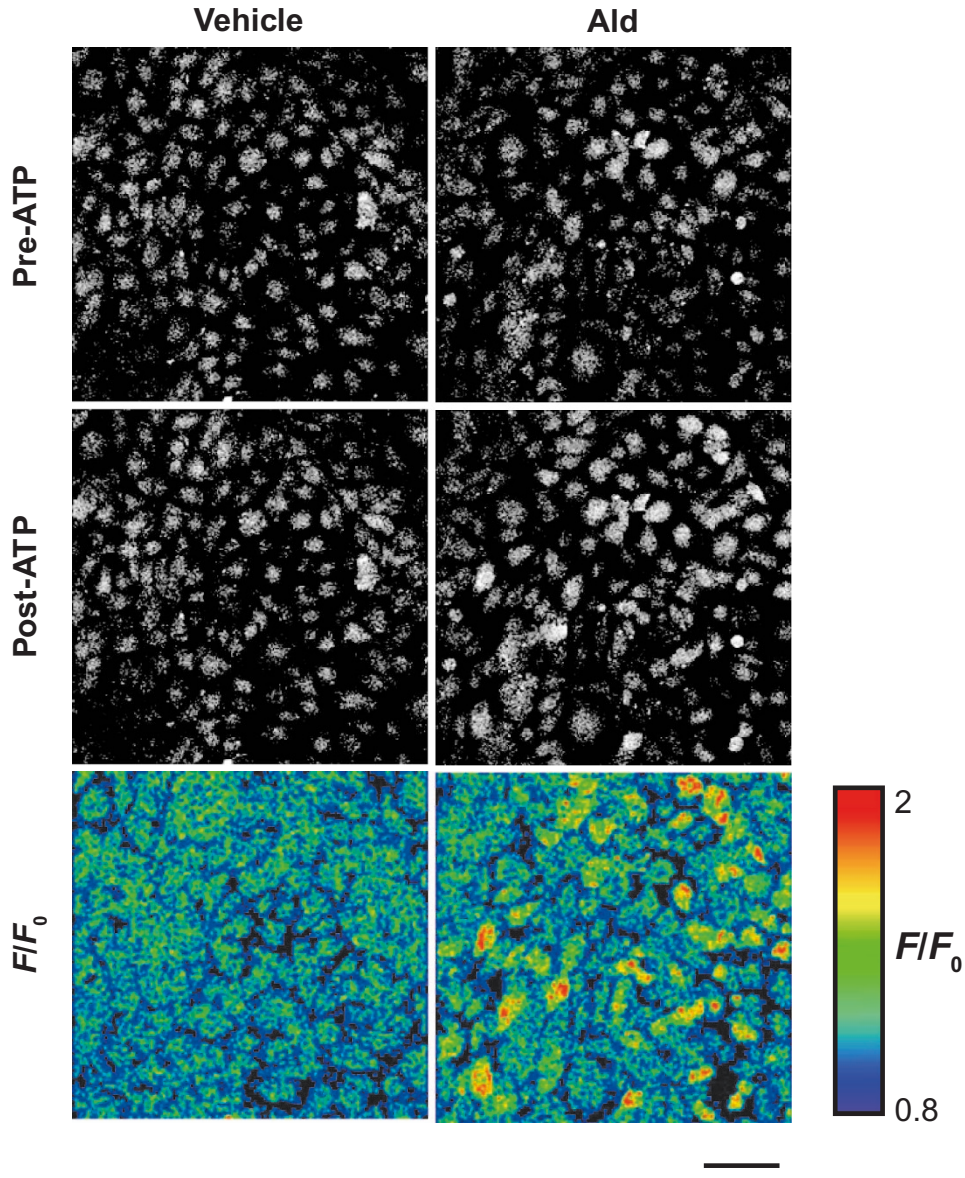

Fig. 1. *Aldosterone enhances ATP-stimulated NO production in ECs. After pretreatment of aldosterone (Ald, 1×10^{-7} mol/L) or vehicle for 5 min, cells loaded with DAF-2 were stimulated with ATP. Gray scale images show cells before and after 1×10^{-5} mol/L ATP application (pre-ATP and post-ATP), and pseudocolored images show the ratio of post-ATP to pre-ATP (F/F_0). Bar: 80 μm. Data represent about 10 experiments.*

(Fura Red) were simultaneously recorded. Fluorescence signals were analyzed with Igor Pro (Wave-Metrics, Portland, USA). Results are presented as the fluorescence ratio ($F_{fluo4}/F_{FuraRed}$) of all cells in the monitored field.

Western Blotting

ECs were cultured in 6 cm dishes (approximately 3×10^6 cells per dish at confluence). Following pre-incubation of the cells with either an inhibitor (1×10^{-5} mol/L eplerenone, 1×10^{-5} mol/L LY294002) or vehicle for 30 min, either aldosterone (1×10^{-7} mol/L) or vehicle was added to the medium for 10 min. After ATP (1×10^{-5} mol/L) or vehicle was applied to the medium, cells were washed with physiological saline and fixed with 10% trichroloacetic acid for 30 min on ice. Fixed cells were scraped into tubes and centrifuged. Pellets were then dissolved in a urea solution (9 mol/L urea, 2% Triton X-100, 1% dithioerythritol) using sonication. Sodium dodecyl sulfate (SDS) sample buffer was added to the samples, which were then subjected to SDS–polyacrylamide gel electrophoresis (PAGE). The proteins were transferred to a PVDF membrane followed by immunoblotting with anti–endothelial

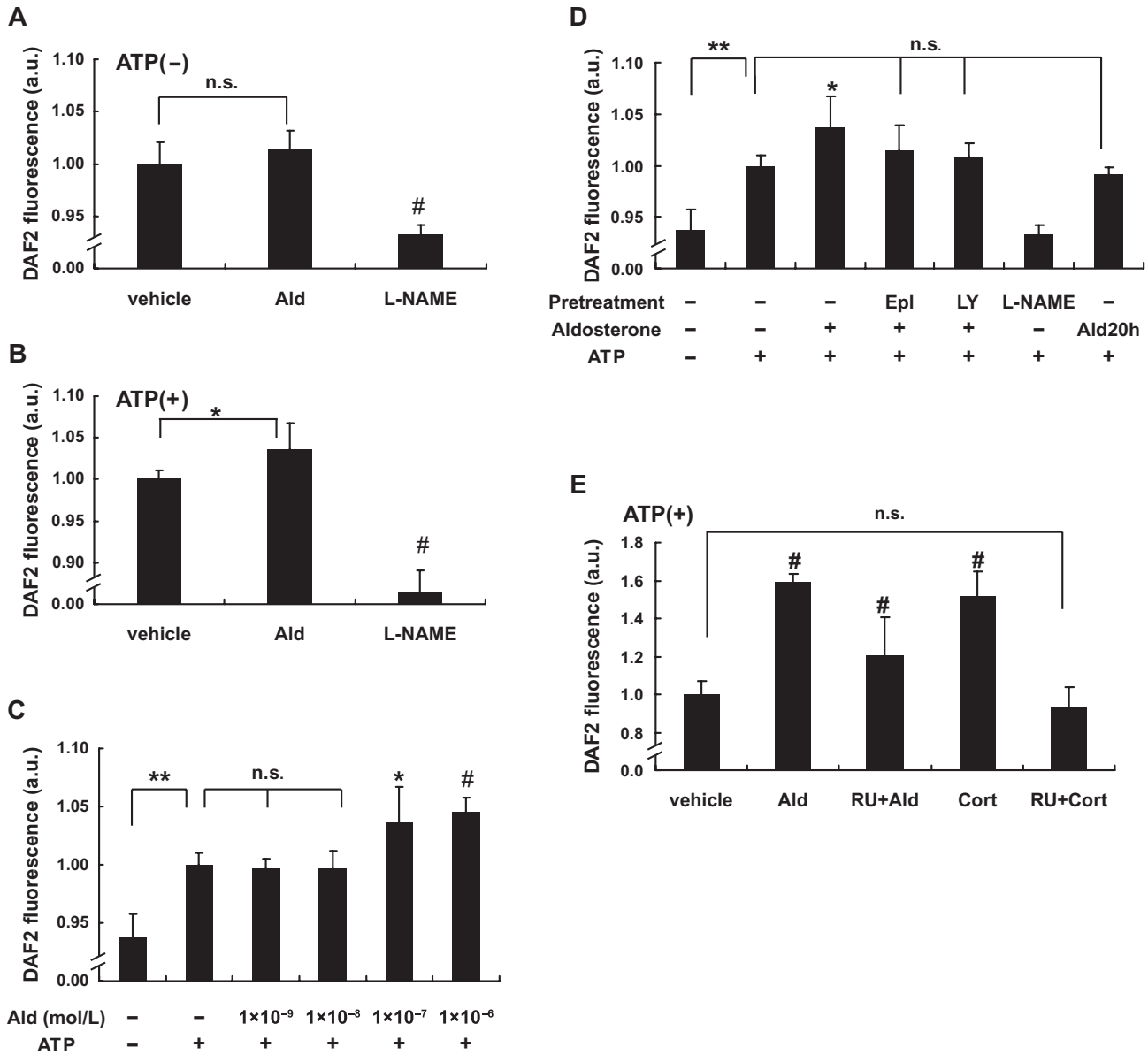

Fig. 2. *Eplerenone and LY294002 inhibit the enhancement of ATP-induced NO production by aldosterone. DAF-2 fluorescence in the culture medium was assayed in the absence (A) or presence (B, C, D, E) of ATP. Enhancement of NO production by treatment with aldosterone (Ald, 1×10^{-7} mol/L) for 10 min was only significant when cells were stimulated with ATP (A, B). L-NAME (1×10^{-4} mol/L), Epl, eplerenone (1×10^{-5} mol/L); LY, LY294002 (1×10^{-5} mol/L); Ald20h, treatment with aldosterone (1×10^{-7} mol/L) for 20 h; RU, RU486 (1×10^{-5} mol/L); Cort, corticosterone (1×10^{-6} mol/L). Data represent mean±SD ($n=5$–6). $^{\#}p<0.001$ vs. vehicle with or without ATP. $^{*}p<0.05$ vs. vehicle with ATP. $^{**}p<0.0001$ vs. vehicle without ATP. n.s., not significant; a.u., arbitrary unit.*

NO synthase (eNOS) or phosphospecific anti-eNOS antibody (pSer^{1177}) (BD Transduction Laboratories, San Jose, USA). Immunoreactive proteins were detected using an ECL kit (Amersham Pharmacia Biotech Inc., Piscataway, USA) according to the manufacturer's protocol.

Recording of Rat Aortic Ring Tension

Eight-week-old male Sprague-Dawley (SD) rats (approximately 240–280 g) were obtained from Tokyo Laboratory Animals Science Co., Ltd. (Tokyo, Japan). The experimental procedures were carried out in accordance with the Guide for the Care and Use of Laboratory Animals published by the

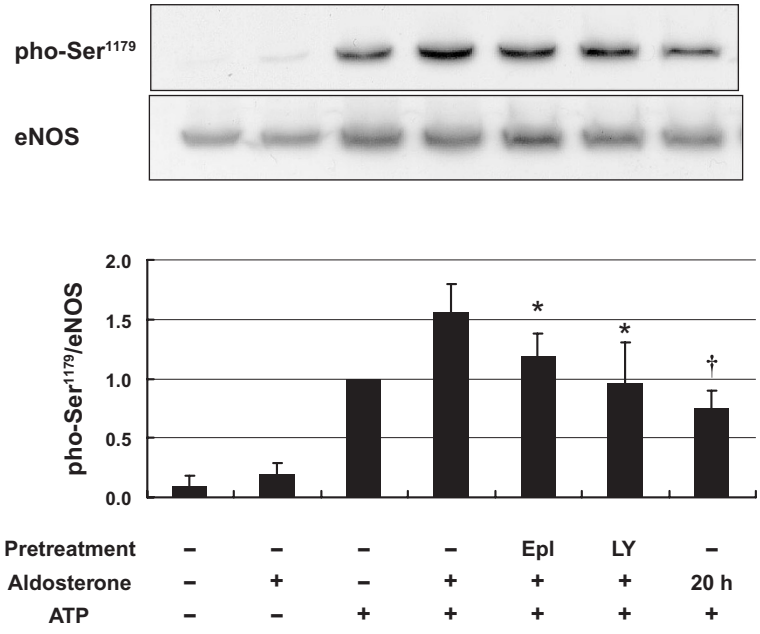

Fig. 3. *Pretreatment with aldosterone facilitates ATP-mediated eNOS phosphorylation at Ser^{1179}. ECs were pre-treated with vehicle (DMSO) or inhibitors (Epl, eplerenone 1×10^{-5} mol/L; LY, LY294002 1×10^{-5} mol/L) for 30 min prior to application of vehicle or aldosterone (1×10^{-7} mol/L). ATP (1×10^{-5} mol/L) was added 10 min after application of vehicle or aldosterone. Cells were fixed 3 min after ATP addition using trichloroacetic acid and then lysed. Ser^{1179} phosphorylation evoked by ATP was enhanced when cells were exposed to aldosterone for 10 min. Pretreatment with Epl or LY prevented Ser^{1179} phosphorylation by aldosterone. Results are expressed after normalization against the ATP group. Data represent mean±SD ($n=4$). *$p<0.05$ vs. aldosterone+ATP, †$p<0.001$ vs. aldosterone+ATP (when normalized against the "aldosterone 20 h" group, the p value between the ATP group and ATP+aldosterone group was calculated as <0.001).*

guidelines of the University of Tokyo Graduate School of Medicine. Rats lightly anesthetized with diethyl ether were sacrificed by collection of whole blood from the abdominal aorta. Then, the thoracic aorta was immediately removed from the body and stripped of connective tissue. In some preparations, the endothelium was mechanically denuded using twisted cotton. The aorta was cut transversally in ring segments with lengths of 4 mm in length. These preparations were performed in Krebs-bicarbonate solution, (112 mmol/L NaCl, 25.2 mmol/L $NaHCO_3$, 4.73 mmol/L KCl, 1.19 mmol/L $MgCl_2$, 1.19 mmol/L KH_2PO_4, 0.9 mmol/L $CaCl_2$, 0.026 mmol/L ethylenediaminetetraacetate [EDTA] disodium, and 11.0 mmol/L glucose), aerated with a mixture of 95% O_2 and 5% CO_2.

The aortic rings were held between a pair of mandolin wires in 10 mL jacketed organ bath chambers filled with Krebs solution; they were maintained at 37°C and aerated continuously with 95% O_2 and 5% CO_2. The lower wire of each ring was fixed in place while the upper was suspended from an isometric transducer (TB-651T; Nihon Kohden Corp., Tokyo, Japan). The output signal of the transducer was digitized by a NI USB-6009 device (National Instruments Corporation, Texas, USA).

While the rings were equilibrated with a resting force of 1 g for 1 h, the medium was exchanged every 15 min. The rings were then pre-contracted twice by application of KCl (60 mmol/L), which were then washed out each time. After subsequent equilibration for 20 min, the rings were contracted with phenylephrine treatment (1×10^{-6} mol/L). After the contractile response reached a plateau, eplerenone (1×10^{-5} mol/L), LY294002 (1×10^{-5} mol/L) or vehicle was added to the medium. Vehicle or aldosterone (1×10^{-9} – 1×10^{-7} mol/L) was then applied for a further 5 min. Five minutes later, cumulative application of acetylcholine (ACh) (3×10^{-9} – 1×10^{-5} mol/L) was initiated to examine endothelium-dependent reactions. To examine endothelium-independent reactions of the smooth muscle cells, sodium nitroprusside was applied cumulatively (1×10^{-10} – 1×10^{-6} mol/L) to endothelium-denuded rings.

Statistical Analysis

Each experiment was repeated at least three times and values were expressed as mean±SD. For comparisons of multiple groups of data, Scheffe's *F* test was used for post-hoc analysis of pairs of groups after initial use of the Kruskal wallis rank test for the NO assay and Western blotting or repeated-measured ANOVA for rat aortic relaxation experiments. $p<0.05$

was considered significant.

Results

Enhancement of NO Production by Aldosterone

First, we examined by confocal microscopy whether aldosterone influences NO production in ECs. NO production was monitored by fluorescence of DAF-2, which is a widely used NO indicator. NO production did not increase for at least 20 min immediately after application of aldosterone (1×10^{-7} mol/L; data not shown), and aldosterone pretreatment did not affect the baseline fluorescence of intracellular DAF-2 (Fig. 1, pre-ATP, Ald *vs*. vehicle). ATP treatment slightly stimulated NO production (Fig. 1, post-ATP, vehicle). Interestingly, ATP induced remarkably larger increases in the NO production of cells pretreated with 1×10^{-7} mol/L aldosterone when compared to those treated with vehicle (Fig. 1, post-ATP, Ald *vs*. vehicle). To quantitatively evaluate the effects of aldosterone, we measured the fluorescence of non-permeable DAF-2 in the culture medium. In the absence of ATP stimulation, pretreatment with 1×10^{-7} mol/L aldosterone did not affect NO accumulation,, which was significantly decreased by L-NAME, an eNOS inhibitor (Fig. 2A). In contrast, aldosterone enhanced ATP-stimulated increases in NO production (Fig. 2B), consistent with the imaging data. The enhancement of ATP-stimulated NO production by aldosterone was seen at above 1×10^{-7} mol/L (Fig. 2C). This rapid effect of aldosterone (1×10^{-7} mol/L) was blocked by eplerenone, an MR antagonist, and LY294002, a PI3K inhibitor (Fig. 2D). Notably, longer treatment with aldosterone (20 h) also abolished the enhancement of NO production (Fig. 2D). Corticosterone, another steroid hormone, enhanced NO production as much as aldosterone. The glucocorticoid receptor (GR) antagonist RU486 almost completely blocked NO enhancement by corticosterone but only partially blocked NO enhancement by aldosterone. These findings suggest that short-term exposure to aldosterone alone does not affect NO production but rather enhances ATP-mediated NO production in ECs by eplerenone-sensitive mechanisms involving PI3K.

Enhanced Phosphorylation of eNOS by Aldosterone

To investigate the mechanisms involved in the enhancement of ATP-stimulated NO production by aldosterone, we next examined eNOS phosphorylation at Ser^{1179} by immunoblotting (Fig. 3). ATP treatment increased phosphorylation of eNOS at Ser^{1179} by about 10-fold over the basal level. Although aldosterone alone barely affected the phosphorylation of eNOS Ser^{1179} (Ser^{1177} for human eNOS), short-term aldosterone exposure significantly enhanced ATP-induced phosphorylation, and this effect was blocked by pre-treatment with eplerenone or LY294002. Treatment with aldosterone for as long as 20 h abolished the enhancement of ATP-induced phosphorylation. The rapid action of aldosterone on ATP-mediated eNOS phosphorylation and its sensitivity to eplerenone and LY294002 agree well with the DAF-2 experiments, suggesting mechanistic links between aldosterone, PI3K, eNOS phosphorylation at Ser^{1179}, and enhancement of ATP-induced NO production.

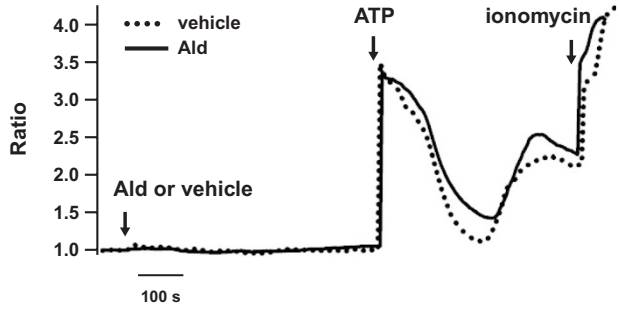

Fig. 4. *Aldosterone does not affect $[Ca^{2+}]_i$ in ECs. Cells loaded with fluo-4 and Fura Red were sequentially stimulated by ATP (1×10^{-5} mol/L) and ionomycin (1×10^{-5} mol/L) in the presence (Ald, solid line) or absence (vehicle, dotted line) of aldosterone, as indicated. Y-axis denotes fluorescence ratio of fluo-4 to Fura Red ($F_{fluo4}/F_{FuraRed}$). Aldosterone treatment evoked no significant increase in $[Ca^{2+}]_i$ for the following 5 min and did not affect ATP- or ionomycin-induced $[Ca^{2+}]_i$ increases. Representative traces are shown for 5 experiments.*

Absence of Effects of Aldosterone on $[Ca^{2+}]_i$

Previous studies have demonstrated nongenomic actions of aldosterone on $[Ca^{2+}]_i$, which is an important regulator of endothelial NO production *via* binding to calmodulin. Thus, we examined whether aldosterone directly affected $[Ca^{2+}]_i$ or modulated ATP-induced $[Ca^{2+}]_i$ increases (Fig. 4). Contrary to previous reports, however, aldosterone alone had no direct effect on $[Ca^{2+}]_i$, which was in agreement with our results showing no direct effect of aldosterone on NO production (Figs. 1 and 2). Subsequent application of ATP and ionomycin rapidly evoked $[Ca^{2+}]_i$ increases, but aldosterone did not affect $[Ca^{2+}]_i$ dynamics including the initial peaks and subsequent decay phases. Accordingly, the enhancement of ATP-induced NO production by aldosterone did not occur through modulation of ATP-induced changes in $[Ca^{2+}]_i$.

Enhancement of ACh-Induced Vasodilation by Aldosterone

Next, to further confirm the rapid effects of aldosterone on ligand-stimulated NO production, we performed experiments using blood vessels. As expected, brief pre-exposure to aldosterone (1×10^{-8} to 1×10^{-7}mol/L) dose-dependently increased the sensitivity to ACh-induced vasorelaxation of

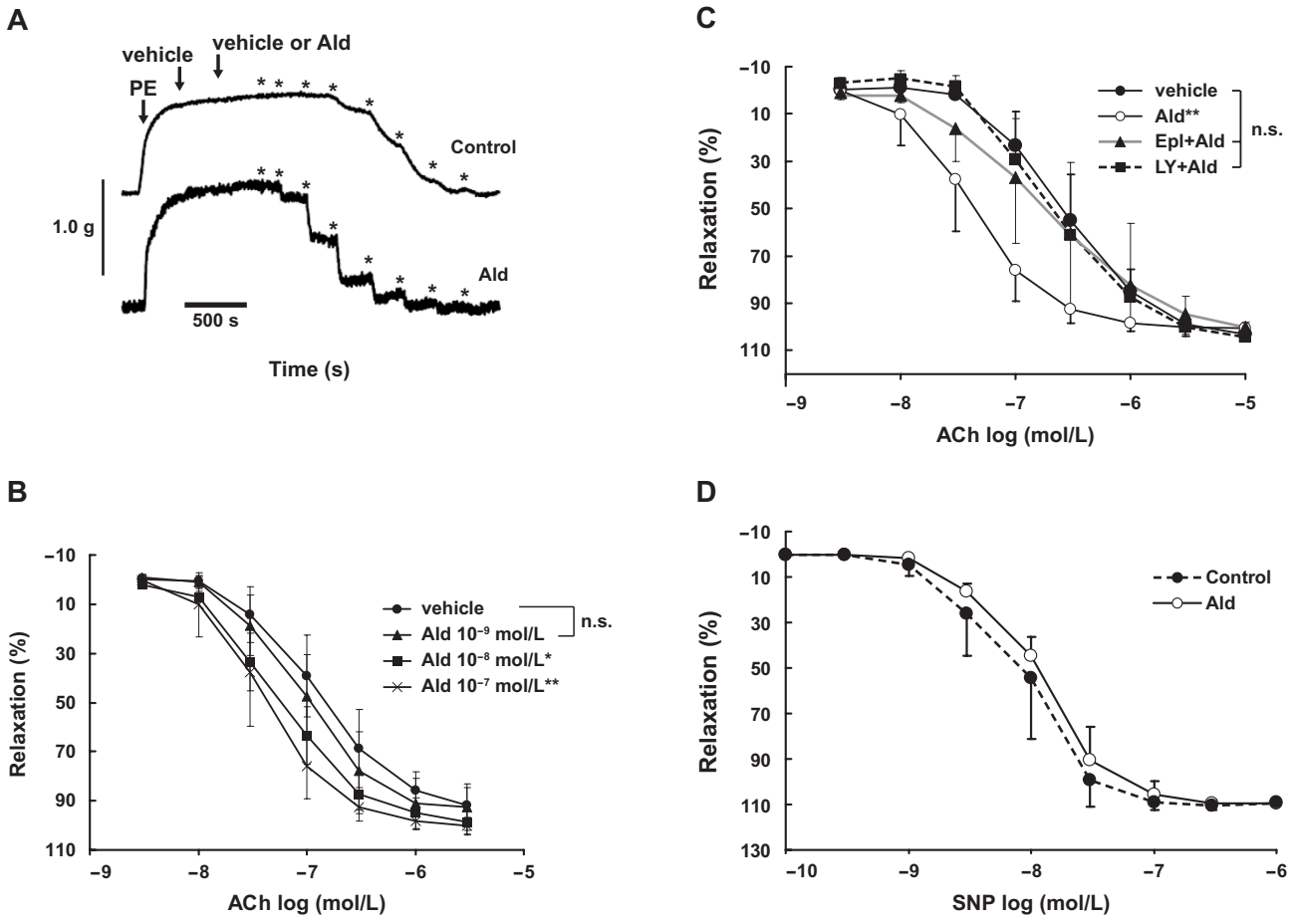

Fig. 5. *Aldosterone enhances ACh-induced relaxation of rat aorta. A: Representative original traces are shown. Cumulative application of ACh (asterisks, 3×10^{-9}, 1×10^{-8}, 3×10^{-8}, 1×10^{-7}, 3×10^{-7}, 1×10^{-6}, 3×10^{-6}, and 1×10^{-5} mol/L) relaxed rat thoracic aortas precontracted with phenylephrine (PE). Aldosterone (Ald, 1×10^{-7} mol/L) or vehicle was administered about 5 min before ACh application. B: Dose-dependent effect of aldosterone on concentration-relaxation curves. The effect of aldosterone was significant at 10^{-7} mol/L (**$p<0.001$) and 10^{-8} mol/L (*$p<0.05$), but not at 10^{-9} mol/L. C: Enhancement of ACh-induced vasorelaxation by aldosterone (*$p<0.001$) was reversed by pretreatment with eplerenone (Epl, 1×10^{-5} mol/L) or LY294002 (LY, 1×10^{-5} mol/L). D: SNP-induced vasorelaxation was not affected by aldosterone in endothelium-denuded aortic rings. Data represent mean±SD ($n=4$–9).*

SD rat aortas precontracted by phenylephrine (Fig. 5A–C). Treatment with 1×10^{-8} and 1×10^{-7} mol/L aldosterone did not affect the maximal response to ACh, but significantly shifted the EC_{50} (50% effective concentration) of ACh from 2.9×10^{-7} mol/L to 5.7×10^{-8} mol/L and 5.1×10^{-8} mol/L, respectively. It should be noted that aldosterone alone had no direct effects on the tone of pre-contracted aortas. In accordance with the cell culture experiments (Figs. 1–3), the enhancement of ACh-induced vasorelaxation by aldosterone was reversed by treatment with eplerenone or LY294002 (Fig. 5C). In contrast, vasorelaxation by sodium nitroprusside (SNP) was not affected by aldosterone in endothelium-denuded aortas, suggesting that the enhancement of vasorelaxation by aldosterone occurs through effects on the endothelium (Fig. 5D).

Discussion

Rapid Action of Aldosterone on ECs

We have demonstrated in this study that short-term exposure of ECs or aortic rings to aldosterone enhances NO production and vasorelaxation induced by Ca^{2+}-mobilizing agonists. Although previously observed rapid effects of aldosterone on endothelium-dependent blood flow in human forearms were discrepant from our findings even under similar experimental conditions, other previous studies have suggested that aldosterone activates NO production, which counteracts vasoconstrictive stimuli such as high K^+ (*18*) and phenylephrine (*9*). Here, we have further investigated a different aspect of the

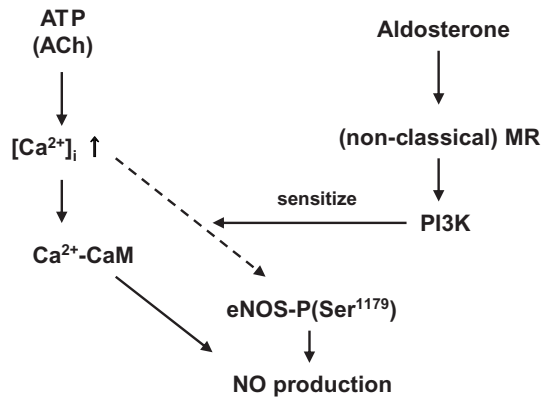

Fig. 6. *Conceivable signaling pathway of the acute action of aldosterone. See Discussion in text.*

vasodilatory role of aldosterone that modifies NO signaling when stimulated by ATP or ACh. Importantly, aldosterone itself could neither mobilize intracellular Ca^{2+} nor enhance phosphorylation of eNOS in ECs, and it did not affect the constriction of vascular tone by phenylephrine. By using membrane-targeted FRET-based Ca^{2+} sensors (*21*), we again confirmed that treatment with aldosterone itself caused no specific subplasmalemmal $[Ca^{2+}]$ increase at all (unpublished observation, Mutoh, Isshiki, and Fujita); this finding conflicts with previous reports (*10*, *22*) for unknown reasons.

The speed of the observed activity of aldosterone suggests that it is probably nongenomic, even though aldosterone is thought to activate a classical MR. Rapid actions of aldosterone may occur *via* classical or non-classical MRs; thus the effects of MR antagonists vary. Haseroth *et al*. (*15*) and Michea *et al*. (*16*) found that aldosterone caused rapid nongenomic increases in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) in skin cells of MR-knockout mice, while another group reported more recently that aldosterone-induced rapid $[Ca^{2+}]_i$ increase in mesenteric arterial cells could be blocked by eplerenone (*16*). If the rapid action of aldosterone on $[Ca^{2+}]_i$ was not mediated through a classical MR, then the observed effect of eplerenone must have also not utilized a classical MR. However, RU28318, another aldosterone antagonist but not spironolcactone, blocked the rapid effect of aldosterone on Na^+/H^+ activity (*11*). Thus, sensitivity to eplerenone may not clearly determine the involvement of the classical MR.

Enhancement of Ca^{2+}-Dependent eNOS Activation by Aldosterone

eNOS is regulated through multiple pathways including protein-protein interactions, phosphorylation, subcellular localization, and several cofactors. ATP and ACh, used in this study as stimuli, are known to mobilize intracellular Ca^{2+} after ligand binding to G-protein–coupled receptors (GPCRs) and subsequent hydrolysis of membrane PIP_2 by PLC to release IP_3. Ca^{2+} released from intracellular Ca^{2+} stores causes Ca^{2+} influx, which is spatially crucial for activating eNOS *via* a Ca^{2+}-calmodulin–dependent pathway (*23*). However, the exact mechanisms involved in eNOS activation by GPCR-associated ligands and $[Ca^{2+}]_i$ remain unclear due to exceptions such as bradykinin (*24*), angiotensin II (*25*, *26*) angiotensin-(1–7) (*27*), and thrombin (*28*). Although the signaling pathways for eNOS activation by GPCRs appear to be ligand-specific, common intermediate molecules and events include Ca^{2+}-calmodulin, Ca^{2+}-dependent kinases such as PKC, and modulation of phosphorylation status. In this study, ATP itself dramatically enhanced eNOS phosphorylation at Ser^{1179} similarly to thrombin, suggesting a role of Ca^{2+}-dependent serine kinases in NO production. However, aldosterone appears to only enhance phosphorylation at Ser^{1179} by LY294002-sensitive PI3K when stimulated by ATP. In contrast, phosphorylation of eNOS at Thr^{495}, another known phosphorylation-dependent regulatory site for eNOS, was not affected by aldosterone under the same experimental conditions (data not shown). Considering that the Akt/PI3K pathway is generally Ca^{2+}-independent, aldosterone-mediated activation of PI3K likely synergizes Ca^{2+}-dependent eNOS phosphorylation at Ser^{1179} by ATP (Fig. 6, schema). The sensitivity of cultured ECs to LY294002 is paralleled by the reversal of aldosterone-mediated enhancement of ACh-induced vasorelaxation by LY294002, suggesting physiological relevance of the observed rapid PI3K-mediated activity. Further investigations are required to clarify the detailed mechanisms.

Multiple Possible Mechanisms Affecting eNOS Activity

The effect of aldosterone in our study was only seen at higher concentrations (1×10^{-8} to 1×10^{-7} mol/L) than normally observed in human. Uhrenholt *et al*. reported effects of aldosterone against vasoconstriction in microperfused rabbit renal afferent arterioles at concentrations ranging widely from 1×10^{-14} to 1×10^{-5} mol/L (*18*). Liu *et al*. also reported that low doses ranging from 1×10^{-12} to 1×10^{-8} mol/L effectively counteracted phenylephrine-induced constriction in rat aortic rings (*9*). The wide range of effective concentrations in different models suggests that multiple pathways with different sensitivities to aldosterone affect eNOS regulation; the mechanism selected likely depends on local endothelial conditions or other environmental elements in different tissues. This speculation is supported by the finding that eNOS activation by aldosterone in phenylephrine-contracted aortic rings of normal rats was less clear in spontaneously hypertensive rats (SHR) (*9*), whereas the enhancement of ACh-induced vasorelaxation by 1×10^{-7} mol/L aldosterone in SD rats was consistently seen even in SHR (unpublished observation, Mutoh, Isshiki, and Fujita). Differences in the experimental redox state may account for some of the discrepancies present between previous findings (*13*).

Chronic Effects of Aldosterone on ECs

The time window appears to be critical for the effects of aldosterone on endothelial function. Nagata *et al*. reported that aldosterone treatment for 16 h impaired eNOS function through decreased levels of the cofactor 5,6,7,8-tetrahydrobiopterin (BH_4) (*29*). We found that enhancement of ATP-induced eNOS phosphorylation seen after 10 min of treatment with aldosterone disappeared when treatment was extended to 20 h. Thus, chronic administration of aldosterone may cause endothelial dysfunction, presumably as the result of its genomic action through MR. This concept is supported by recent clinical trials demonstrating beneficial cardiovascular effects of MR antagonists in certain populations. It is of great interest to clarify the mechanism underlying the time-sensitive effects of aldosterone on endothelial function and the cardiovascular system.

Physiological Significance

The pathophysiological significance of the nongenomic action of aldosterone is currently unclear. The lowest concentration of aldosterone (1×10^{-8} mol/L) that we found effective in our *ex vivo* experiment was higher than the physiological range, and the possible involvement of the GR cannot be neglected. Corticosterone enhanced NO production, and this effect was completely blocked by RU486, a GR antagonist (Fig. 2E). Notably, the blockade of aldosterone-mediated NO enhancement by RU486 was only partial, suggesting that at least some effects of aldosterone are exerted independently of GR. In summary, we have shown direct evidence that aldosterone acts acutely on the endothelium as a vasodilator and that it enhances ligand-stimulated NO production by the PI3K pathway but not by generation of increased $[Ca^{2+}]_i$.

Perspectives

Many issues must be further addressed regarding the nongenomic actions of aldosterone, including their association with the development of cardiovascular diseases such as hypertension or chronic heart disease, MR signaling associated with the redox state, aldosterone production in local tissues, and temporal and functional coordination with genomic actions. The present work provides a new insight into a regulatory system for endothelial function as well as some clues for reconciling the currently conflicting data regarding the rapid effects of aldosterone on the cardiovascular system.

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