Aldosterone and Vascular Endothelium

Molecular Mechanism of the Inhibitory Effect of Aldosterone on Endothelial NO Synthase Activity

Daisuke Nagata, Masao Takahashi, Kuniko Sawai, Tetsuya Tagami, Takeshi Usui, Akira Shimatsu, Yasunobu Hirata, Mitsuhide Naruse

Abstract—Although the proinflammatory and profibrotic actions of aldosterone (Aldo) on the vasculature have been reported, the effects and molecular mechanisms of Aldo on endothelial function are yet to be determined. We investigated how Aldo regulates endothelial NO synthase (eNOS) function in human umbilical vein endothelial cells (HUVECs). HUVECs were incubated for 16 hours with Aldo $10^{-7}$ mol/L. The concentration of reactive oxygen species was estimated by measuring 2',7'-dichlorodihydrofluorescein diacetate chemiluminescence. Signal transduction was estimated by Western immunoblots. Real-time RT-PCR was performed to measure expression of transcripts of endogenous GTP cyclohydrolase-1 and components of reduced nicotinamide-adenine dinucleotide phosphate oxidase. To eliminate the possible effect of the glucocorticoid receptor (GR) and to emphasize the role of mineralocorticoid receptor, we used GR small interfering RNA and knocked down GR expression in several experiments. NO output was estimated by intracellular cGMP concentration. Reactive oxygen species production increased significantly in Aldo-treated HUVECs but was abolished by pretreatment with eplerenone. Transcripts of p47phox were increased by Aldo treatment. Vascular endothelial growth factor–induced eNOS Ser 1177 but not Akt Ser 473 phosphorylation levels were reduced significantly by pretreatment with Aldo. Pretreatment with either eplerenone or okadaic acid restored phosphorylation levels of eNOS Ser 1177 in Aldo-treated cells, suggesting that protein phosphatase 2A was upregulated by Aldo via mineralocorticoid receptor. The decrease in NO output caused by Aldo pretreatment was reversed significantly by 5,6,7,8-tetrahydrobiopterin, GTP cyclohydrolase-1 overexpression, or p47phox knockdown. These results suggest that Aldo inhibits eNOS function through bimodal mechanisms of 5,6,7,8-tetrahydrobiopterin deficiency and protein phosphatase 2A activation. (Hypertension. 2006;48:165-171.)

Key Words: aldosterone  ■  nitric oxide synthase

Aldosterone (Aldo) is the major hormone controlling sodium reabsorption. Aldo elevates blood pressure as a result of volume expansion after sodium absorption in the renal distal tubules. Whereas this increase in blood pressure has unfavorable effects on the vasculature, patients with primary aldosteronism (PA) have been believed to have a relatively good prognosis, because renin activity is usually suppressed in these patients. However, numerous studies have shown unexpectedly that PA is associated with an increased prevalence of cardiovascular complications, such as aortic dissection, myocardial infarction, or stroke.1-3 In addition, recent clinical trials, the Randomized AlDactone Evaluation Study (RALES) and Eplerenone Post-acute myocardial infarction Heart failure Efficacy and Survival Study (EPHESUS), showed that mineralocorticoid receptor (MR) antagonists improved the prognosis of chronic heart failure patients even at doses below the threshold that caused significant renal effects.4,5 This finding suggested that MR antagonism may have a direct protective effect on the cardiovascular system. Furthermore, there is a large body of evidence that Aldo induces inflammatory changes in the vasculature leading to deterioration in vascular function. Recent evidence suggests that reactive oxygen species (ROS) are the major mediator of vascular inflammation and endothelial dysfunction.6-8 Multiple aspects of Aldo and MR functions in epithelial cells and nonepithelial cells were reviewed in detail by Fuller and Young9 and Funder.10 Although in vivo studies have shown that Aldo induces unfavorable changes in the vasculature, the effect of Aldo on endothelial cells, especially on the function of endothelial NO synthase (eNOS), remains controversial. Laursen et al11 showed that angiotensin II–induced ROS production may alter endothelium-dependent vascular relaxation as a result of interaction between superoxide and NO and also as a consequence of interactions between peroxynitrite and the eNOS cofactor, 5,6,7,8-tetrahydrobiopterin (BH$_4$). This observation strongly supports the possibility that oxidation of BH$_4$ by ROS may be a pathogenic cause of eNOS “uncoupling.” We, therefore, speculated that this mechanism may also be in-
volved in the pathophysiology of Aldo-induced endothelial dysfunction. In the present study, we attempted to clarify the effects of Aldo on eNOS function in endothelial cells.

Methods

Materials
Phospho-eNOS (Ser 1177), phospho-eNOS (Thr 495), and phospho-Akt (Ser 473) were purchased from Cell Signaling Technology. Actin, eNOS, and Akt antibody were purchased from Santa Cruz Biotechnology. Eplerenone was a generous gift from Pfizer. (6R)-5,6,7,8-Tetrahydro-1-bioprotein dihydrochloride and other chemical reagents were purchased from Wako Pure Chemicals, human umbilical vein endothelial cells (HUVECs) and Humedia EG growth medium from Kurabo, recombinant human vascular endothelial growth factor (VEGF) 121 from R&D Systems, and control green fluorescent protein expression vector from Invitrogen.

Construction of GTP Cyclohydrolase-1 Expression Vector
Details are available in Supplementary Text I available at http://www.hypertensionaha.org.

Cell Culture and Vector Transduction
HUVECs were cultured in Humedia EG with 2% FBS, 100 U/mL penicillin, and 100 µg/mL streptomycin. For the experiments involving gene transduction, either GTP cyclohydrolase-1 (GCH1) expression vector or control green fluorescent protein expression vector were transfected at 4 µg for 5 × 10^5 cells with the PolyMag Magnetofection kit (OZ Bioscience) according to the manufacturer’s instructions. After an incubation period of 2 days, the medium was replaced with Humedia basal medium containing 0.1% FBS to reduce the effects of stimulation by serum mitogens. After 8 hours of incubation in low-serum medium, the vector-transfected HUVECs were treated with Aldo (10^{-7} mol/L). When treated with inhibitors such as eplerenone (10^{-3} mol/L), N^6-nitro-L-arginine methyl ester ([l-NAME] 2 mmol/L), or okadaic acid (10^{-5} mol/L), the cells were incubated with the indicated chemicals 1 hour before stimulation with aldosterone.

Real-Time RT-PCR
All of the primers used for quantitative real-time RT-PCR (NOX1, NOX2, NOX4, p22phox, p40phox, p47phox, p67phox, GCH1, and GAPDH) were purchased from Takara. The experiments were performed using SYBR One-Step quantitative RT-PCR kits (Invitrogen) according to the manufacturer’s instructions, with GAPDH as the internal control.

Small Interfering RNA Construction and Transfection
Glucocorticoid receptor (GR) and p47phox small interfering RNA (siRNA) were constructed, using as a reference the cDNA sequence obtained from the Whitehead Institute web-based computer program (http://jura.wi.mit.edu/bioc/siRNAext/). Details are described in Supplementary Text II (available online).

Measurement of ROS
Intracellular production of ROS in HUVECs after incubation with Aldo and/or eplerenone was measured by 2',7'-dichlorodihydrofluorescein diacetate ([DCF] 10 µmol/L, Wako Pure Chemicals) according to the manufacturer’s instructions. HUVECs were loaded with DCF for 30 minutes at 22°C, washed 3 times in PBS, and then placed on a laser scanning confocal microscope CSU21 (Yokokawa Electric), equipped with a high-resolution digital CCD camera, ORCAII-ER (Hamamatsu Photonics). Images were collected by single rapid scans with identical parameters being used for all of the samples. Fluorescence intensity data were quantified in 4 independent experiments.

Western Blot Analysis
Western blot analysis was carried out as described previously. Briefly, cell lysates were extracted with Nonidet P-40lysis buffer (50 mmol/L of Tris-HCl [pH 8.0], 150 mmol/L of NaCl, 1% [NP-40], 2 µg/mL of aprotinin, 2 µg/mL of leupeptin, 1 mmol/L of PMSE, and 1 mmol/L of sodium orthovanadate) followed by SDS-PAGE (25 µg of protein per lane). For immunoblot analysis of the dimeric form of eNOS, samples were subjected to 6% to 9% gradient polyacrylamide gels without preheating, and the temperature of the gels was maintained at 4°C during electrophoresis (low-temperature SDS-PAGE). To separate membrane and cytosolic protein fraction and evaluate p47phox translocation, we used the ProteoExtract Subcellular Proteome Extraction kit (Calbiochem) according to the manufacturer’s instructions. The membranes were immunoblotted with the indicated antibodies at 1:250 to 1:1000 dilutions followed by incubation with the secondary antibody conjugated with horseradish peroxidase at 1:6000 dilution. The ECL PLUS Western Blotting Detection kit (Amersham Pharmacia Biotech) was used for detection. The density of the bands was quantified using the NIH Image program. Each experiment was repeated 3 to 4 times.

cGMP Assay
To evaluate NO output from HUVECs, intracellular cGMP concentration was measured using a cGMP enzyme immunoassay system (R&D Systems). Details are described in Supplementary Text III (available online).

Protein Phosphatase 2A Assay
Protein phosphatase (PP) 2A activity was measured in HUVECs treated with Aldo and/or other reagents, such as eplerenone (10^{-5} mol/L) and okadaic acid (10^{-4} mol/L) for 16 hours after incubation in low-serum medium. We performed this assay using PP2A immunoprecipitation phosphatase assay kit (Upstate Biotechnology) according to the manufacturer’s instructions.

Statistical Analyses
Values are expressed as mean±SEM. Statistical comparisons were performed using ANOVA with Scheffe’s F procedure for posthoc analysis. P<0.05 was considered as statistically significant.

Results

Effects of Aldo on ROS Production in HUVECs
HUVECs stimulated for 16 hours with Aldo were shown to produce ROS in a dose-dependent manner (Figure 1A). Pretreatment with eplerenone 10^{-5} mol/L significantly suppressed the Aldo-induced increase in ROS production (Figure 1A). Figure 1B shows the semiquantitative analyses of the DCF intensities.

Effects of Aldo on Transcription of p47phox and Other NADPH Oxidase Components
Aldo at 10^{-9} to 10^{-7} mol/L increased p47phox transcription significantly in a dose-dependent manner (Figure 2A). However, transcription levels of NOX1, NOX2, NOX4, p22phox, p40phox, p47phox, or p67phox were not changed by Aldo stimulation (data not shown). Eplerenone 10^{-5} mol/L significantly inhibited p47phox upregulation caused by Aldo (Figure 2A). GR knockdown did not change the levels of Aldo-induced p47phox transcripts compared with scrambled siRNA controls (Figure 2B).

Effects of Aldo on Translocation of p47phox to the Plasma Membrane
We also showed that Aldo significantly increased the membrane localization of p47phox protein. However, eplerenone
significantly inhibited membrane-localized p47phox (Figure I, available online).

Effects of Aldo on NO Production in HUVECs
The effects of Aldo on NO production were investigated by measuring intracellular cGMP concentration in HUVECs with or without stimulation of VEGF (100 ng/mL). Pretreatment with Aldo $10^{-7}$ mol/L for 16 hours significantly inhibited NO output in HUVECs stimulated with VEGF (Figure 3A). The effects of Aldo were reversed by eplerenone $10^{-5}$ mol/L (Figure 3B). In unstimulated HUVECs, Aldo treatment did not result in significant changes in NO output, with output levels being approximately the same as that caused by eNOS-inhibited conditions with L-NAME.

Effects of Aldo on Ser 1177 Phosphorylation of eNOS
Stimulation with VEGF (100 ng/mL) for 30 minutes increased phosphorylation of both eNOS Ser 1177 and Akt Ser 473 of eNOS to $\approx 3$ times the levels seen without stimulation. Pretreatment with Aldo ($10^{-7}$ mol/L) for 16 hours significantly inhibited the VEGF-stimulated increase in the phosphorylation of eNOS Ser 1177 (Figure 4A). GR knockdown did not alter the effect of Aldo on eNOS phosphorylation (Figure 4A). Eplerenone $10^{-5}$ mol/L or okadaic acid $10^{-8}$ mol/L significantly reversed the inhibitory effect of Aldo on eNOS Ser 1177 phosphorylation, whereas Akt Ser 473 phosphorylation remained unchanged (Figure 4B). The phosphorylation levels of eNOS Thr 495 did not change under the conditions tested. Figure 4C shows the quantitative analysis of relative phosphorylation levels of eNOS Ser 1177 and Akt Ser 473 in Figure 4B (n=4).

Effects of Aldo on eNOS Dimerization
We performed low-temperature SDS-PAGE and immunoblotting to investigate eNOS dimerization. There was no difference in eNOS expression between control and Aldo-treated cells using conventional SDS-PAGE, but low-temperature SDS-PAGE showed that the eNOS dimer/monomer ratio decreased when treated with Aldo (Figure 5A). Eplerenone or BH$_4$ cotreatment significantly reversed
this inhibition. Figure 5B shows the quantitative analysis of
the dimmer/monomer ratio of eNOS in Figure 5A.

### PP2A Activity Induced by Aldo

Aldo treatment for 16 hours significantly activated PP2A.
Eplerenone or okadaic acid cotreatment significantly inhibited this activation (Figure II, available online).

### Effects of Aldo on GCH1 Transcriptional Level

We investigated whether Aldo regulated the gene of GCH1, which catalyzes the conversion of GTP to D-erythro-7,8-dihydronopterin triphosphate, the first and rate-limiting step in BH4 biosynthesis. Aldo at concentrations between $10^{-9}$ and $10^{-7}$ mol/L did not change GCH1 transcription levels after 16 hours of incubation (Supplementary Figure III). In addition, there was no significant change in GCH1 transcription levels after incubation with Aldo ($10^{-7}$ mol/L) for 2, 4, and 8 hours (data not shown).

### Effects of BH4, GCH1 Overexpression, or p47phox Knockdown on NO Output Inhibited by Aldo

The additional BH4 and GCH1 overexpression by the GCH1-expression vector partially but significantly reversed the Aldo-induced decrease in NO production (Figure 6A). In addition, p47phox knockdown using siRNA (75% reduction compared with control; Figure 6B) also reversed the inhibitory effects of Aldo on eNOS function (Figure 6A).

### Discussion

In this study in HUVECs, we showed that Aldo upregulated intracellular ROS production using DCF fluorescence. We also demonstrated that Aldo increased NADPH oxidase component p47phox transcription using real-time RT-PCR. Although ROS may derive from mitochondria, xanthine oxidase, cyclooxygenase, NO synthase, heme oxygenases, or peroxidases, it has been shown frequently that NADPH oxidases are the primary producers of ROS in vascular tissues.14–16 Previous publications have suggested that ROS produced by NADPH oxidase mediate many angiotensin II effects in the cardiovascular system.17,18 It was reported recently that Aldo also increases NADPH oxidase activity in the vasculature.19,20 In the NADPH oxidase system, cytosolic component p47phox was shown to have a pivotal role in the regulation of enzymatic activity. Landmesser et al21 reported that the hypertensive response and production of vascular superoxide was markedly blunted in p47phox knockout mice. Nishiyama et al22 have also shown that Aldo induced NADPH oxidase activation and membranous translocation of p47phox and p67phox in cultured rat mesangial cells. In the present study, we showed clearly using real-time RT-PCR that Aldo increased p47phox transcription (Figure 2A). We also observed that Aldo increased translocation of p47phox to the plasma membrane.
fraction (Figure I). These observations show that Aldo increases ROS production in HUVECs by activating NADPH oxidase, mainly via p47phox-transcriptional and-translocational regulation. We showed that, although p47 phox transcription was almost completely inhibited by eplerenone (Figure 2), ROS production was not suppressed to the basal level (Figure 1). We could not detect ROS production within 2 hours of stimulation by Aldo (data not shown). These results suggested that Aldo-induced ROS production and redox-sensitive eNOS dysfunction were mainly mediated by a genomic mechanism. However, we could not rule out the possibility that nongenomic action of Aldo, which cannot be inhibited by eplerenone, partially mediated ROS production. Furthermore, there is a possibility that other minor ROS sources activated by Aldo are not inhibited by eplerenone.

In the absence of either L-arginine or BH₄, eNOS produces superoxide and hydrogen peroxide instead of NO.²³,²⁴ This phenomenon has been referred to as uncoupling. It has been shown in the vasculature of apolipoprotein E-deficient mice that upregulation of ROS production and subsequent oxida-
tion of BH4 by peroxynitrite may lead to uncoupling of eNOS.\textsuperscript{11} The present study demonstrated that eNOS function was negatively regulated by Aldo via 2 distinct mechanisms. The first mechanism was through oxidation of BH4 and uncoupling of eNOS because of a deficiency in its cofactor. This is the first report that Aldo has an action similar to angiotensin II, which results in suppression of eNOS function via an NO synthase uncoupling mechanism, induced by BH4 deficiency. Bendall et al\textsuperscript{25} and other groups have shown that the eNOS dimer/monomer ratio serves as a marker of eNOS uncoupling. We performed low-temperature SDS-PAGE and showed that Aldo attenuated eNOS dimer formation (Figure 5). Furthermore, eplerenone or BH4 cotreatment reversed this inhibition (A). B, Quantitative analysis of the dimmer/monomer ratio of eNOS presented in A (n=3). Bar, mean±SEM; Eplr indicates eplerenone 10\textsuperscript{-5} mol/L; *P<0.01 vs Aldo.

Several studies have shown that Aldo acts as a vasodilator because of its role as a positive regulator of eNOS through Akt activation.\textsuperscript{27,28} Although these observations seem to be inconsistent with the findings of our study, the experimental conditions used in these earlier studies were completely different from our methodology. Because the Aldo stimulation time was relatively short (a couple of minutes) in these other studies, it is possible that Aldo may have caused vasodilation as a result of redox-sensitive Akt activation or, alternatively, by an unknown non-genomic mechanism. Uhrenholt et al\textsuperscript{29} also suggested that the detrimental effects of Aldo on cardiovascular function may be via a genomic effect that is balanced by nongenomic activation of eNOS in healthy individuals.

Whenever the function of Aldo through MR is investigated, it is important to consider the possibility of a cross-reaction between Aldo and GR. Because there is evidence that GR binds Aldo at almost the same affinity as cortisol and that GR mediates the effects of Aldo,\textsuperscript{30,31} we used an siRNA technique to reduce GR expression and thereby eliminate the effects of Aldo on the receptors. GR knockdown did not cause any difference in phosphorylation levels of eNOS in the HUVECs compared with controls. Furthermore, we found that the MR-specific antagonist, eplerenone, significantly blocked the inhibitory effects of Aldo on eNOS function. This suggests that MR was the main receptor mediating the pro-oxidative effect of Aldo in our investigations.

In summary, this study demonstrated the critical roles of eNOS uncoupling and PP2A activation induced by Aldo in HUVECs. We consider it important that further studies investigate the molecular mechanism underlying Aldo-induced endothelial dysfunction, because this knowledge may lead to novel strategies for the prevention of atherosclerosis.
Perspectives
It is well recognized that Aldo induces cardiovascular damage, such as endothelial dysfunction or perivascular fibrosis, but the exact mechanisms of these effects are not well established. This study has shown that Aldo downregulates eNOS activity via ROS production and Ser 1177 dephosphorylation in an MR-dependent manner. Future studies are needed to ascertain whether MR blockade could inhibit oxidative stress and improve endothelial function in in vivo models.

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Disclosures
None.

References

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Text Supplements

<Supplementary Text 1>

Construction of GCH1-expression Vector

GCH1 expression vector was constructed as follows. Using human umbilical vein endothelial cells (HUVECs) mRNA as templates, human GCH1 cDNA (Genbank Accession No. NM_000161) was subcloned by RT-PCR using an upper primer 5’-CCCAAGCTT-GAGTTAGCCGCAGACCTCGAAG-3’ (nucleotides 96-118) and a lower primer 5’-CCCGAATTC-TCGGCAACCAACGCACACAC-3’ (nucleotides 946-927). The RT-PCR product fragment was treated by HindIII/ EcoRI and ligated into HindIII/ EcoRI-digested pcDNA3.1/Zeo(+) vector (Invitrogen).

<Supplementary Text 2>

siRNA construction and transfection

GR

GenBank Accession No. X03225, position 1160-1182,
sense: 5’-CAGCAGGAUCAGAAGCCUA-tt-3’,
antisense: 5’-UAGGCUUCUGAUCCUGCUG-tt-3
GenBank Accession No. NM_000265, position 76-98,
sense: 5’-GCACUAUGUGUACAUAGUC-tt-3’,
antisense: 5’-GAACAUGUACACAUAGUGC-tg-3

These primers were synthesized by Greiner Bio-One (Frickenhausen, Germany).

Negative control scrambled siRNA (Negative Control #1 siRNA) was purchased from
Ambion (Austin, TX). The siRNA (12 pmol/well) was transfected to HUVECs (5 x 10^5
cells/well) with 6µl/well TransIT-TKO (Mirus Bio, Madison, WI), according to the
manufacturer’s instructions. The knockdown ratio was evaluated by Western blot
analyses.

<Supplementary Text 3>

cGMP Assay

After incubation in low serum medium for 8 hrs, the cells were incubated in a
serum free medium for 16 hrs with Aldo or the other reagents such as BH4 (1 mmol/L).
In GCH1- or p47phox-siRNA- transfected cells experiments, transfection was
performed 2 days before the low serum medium change. VEGF (100ng/mL) was then
added to the cultures in the absence or presence of the NO synthase inhibitor, L-NAME
(2 mmol/L). Each experiment was performed on 4 independent samples, with 3 sets of separate experiments being performed.

**Supplementary Figure Legends**

**Supplementary Figure 1**

Membrane-localized p47phox protein measured by Western immunoblotting. Aldo $10^{-7}$ mol/L significantly increased membrane localization of p47phox protein, but eplerenone $10^{-5}$ mol/L significantly inhibited membrane-localized p47phox. The cytosolic fraction of p47phox did not change after Aldo treatment. Total cell lysate immunoblotted with actin shows that the amount of loading protein was the same. Each bar represents the mean ± SEM (N=4), *:p< 0.01 vs aldosterone -/ eplerenone -, †:p<0.01 vs aldosterone +/- eplerenone -

**Supplementary Figure 2**

Aldo treatment increased PP2A activity in HUVECs. Eplerenone or okadaic acid co-treatment inhibited this activation. Four independent experiments were performed. Eplr: eplerenone $10^{-5}$ mol/L, OA: okadaic acid $10^{-8}$ mol/L. Each bar represents the mean
±SEM, *: p<0.01 vs aldosterone -

Supplementary Figure 3

GCH1 transcriptional levels in Aldo-treated HUVECs. Real time RT-PCR shows that Aldo at concentrations between $10^{-9}$ to $10^{-7}$ mol/L did not change endogenous GCH1 transcription levels after 16 hours of incubation.
Supplemental Figure 1 Nagata D