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Aldosterone inhibits endothelial morphogenesis and angiogenesis through the downregulation of vascular endothelial growth factor receptor-2 expression subsequent to peroxisome proliferator-activated receptor gamma

Miki Fujii[a], Isao Inoki[a], Makoto Saga[a], Norihiro Morikawa[a], Ken-ichiro Arakawa[a], Satoru Inaba[a], Kazuaki Yoshioka[b], Tadashi Konoshita[a], Isamu Miyamori[a,*]

[a] Third Department of Internal Medicine, Faculty of Medical Sciences, Fukui University, 23-3 Matsuokashimoaizuki, Eiheiji-cho, Yoshida-gun, Fukui 910-1193, Japan
[b] Department of Physiology, Kanazawa University Graduate School of Medicine, 13-1 Takara-machi, Kanazawa, Ishikawa 920-8640, Japan

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ABSTRACT

Angiogenesis plays a pivotal role in cardiovascular diseases such as ischemic heart disease, limb ischemia and heart failure, and has recently been shown to mediate various biological activities related to the pathogenesis of these diseases. In the present study, we evaluated the role of aldosterone in angiogenesis. Tube formation assay on Matrigel using human umbilical vein endothelial cells (HUVEC) revealed that aldosterone inhibited endothelial morphogenesis in a manner sensitive to eplerenone, a selective mineralocorticoid receptor antagonist. The anti-angiogenic effect of aldosterone was further confirmed by an *in vivo* angiogenesis assay using a Matrigel plug model in mice. Reverse transcription-mediated polymerase chain reaction and immunoblotting demonstrated that aldosterone downregulated the expression levels of vascular endothelial growth factor receptor-2 (VEGFR-2) and peroxisome proliferators-activated receptor gamma (PPAR gamma). VEGFR-2 expression was found to be enhanced in response to PPAR gamma activation by troglitazone, and attenuated by GW9662, a specific antagonist of PPAR gamma. In the tube formation assay, endothelial morphogenesis was stimulated by troglitazone, and inhibited by GW9662, indicating that PPAR gamma activation mediates positive regulation of angiogenesis through enhancement of VEGFR-2 expression. These data suggest that aldosterone inhibits angiogenesis through VEGFR-2 downregulation, subsequent to, at least in part, attenuation of PPAR gamma expression. The present findings provide a new insight into the possible therapeutic application of mineralocorticoid receptor blockade to various cardiovascular diseases.

1. Introduction

Neovascularization in living tissue consists of two different cellular mechanisms. One is angiogenesis, the formation of new capillary networks from pre-existing vessels, and the other is vasculogenesis, which involves differentiation and incorporation of circulating bone marrow-derived endothelial precursor cells into capillaries [1–3]. Angiogenesis plays a pivotal role in various pathophysiological conditions such as embryogenesis, inflammation, tumor growth and metastasis, and diabetic retinopathy [4–6]. In cardiovascular diseases including ischemic heart disease and limb ischemia, angiogenesis also plays an important role, since it provides a means of rescue for hypoperfused tissues [7]. Moreover, recent studies have suggested that reduction of systolic function in heart failure results from loss of a proportional increase in capillary density along with hypertrophy of cardiac myocytes [8].

Many growth factors and cytokines, produced in response to hypoxic or proinflammatory stimuli, have been reported to stimulate angiogenesis [3]. Among them, vascular endothelial growth factor (VEGF) is a prominent mitogen that stimulates endothelial proliferation and differentiation mediated mainly by its specific receptor, VEGFR-2 [9]. Regulation of VEGF and VEGFR-2 expression would be therapeutically beneficial for control of diseases in which angiogenesis plays a pathogenetic role [10,11]. Although attempts have been made to clarify the mechanisms involved in angiogenesis, and many growth factors and cytokines have been reported to regulate it, their efficacies have not been fully established in a clinical setting.

Aldosterone is a mineralocorticoid that mediates salt and water resorption in the distal tubules of the kidney. Besides its classical function, aldosterone has recently been shown to mediate various other biological functions through the mineralocorticoid receptor (MR) in other organs. For example, aldosterone stimulates cardiac

* Corresponding author. Tel.: +81 776 61 3111; fax: +81 776 61 8111.
E-mail address: miyamori@u-fukui.ac.jp (I. Miyamori).

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hypertrophy and fibrosis [12]. Endothelial dysfunction and vascular injury are also reported to result from elevated oxidative stress and production of proinflammatory cytokines by aldosterone [13]. Furthermore, clinical trials have demonstrated that mineralocorticoid receptor blockade reduces mortality in patients with heart failure after acute myocardial infarction [14]. Thus, aldosterone undoubtedly exerts adverse effects in the pathogenesis and progression of various cardiovascular diseases; however, the effects of aldosterone on angiogenesis remain to be clarified.

In the present study, we examined the roles of aldosterone in angiogenesis *in vitro* and *in vivo*. We found that aldosterone inhibits endothelial morphogenesis and angiogenesis *in vivo* by down-regulating the expression of VEGFR-2, a receptor for VEGF, and peroxisome proliferator-activated receptor gamma (PPAR gamma).

2. Materials and methods

2.1. Materials

Aldosterone, troglitazone (a PPAR gamma agonist) and GW9662 (a PPAR gamma antagonist) were purchased from Sigma. The mineralocorticoid receptor-selective antagonist, eplerenone, was a gift from Pfizer. Recombinant human VEGF was obtained from R&D Systems. Matrigel was purchased from BD Biosciences (Tokyo, Japan).

2.2. Tube formation assay

The tube formation assay was performed on the surface of growth factor-reduced Matrigel, as described previously [10]. Five hundred microliters of Matrigel was dispensed into a 24-well plate and allowed to gel at 37 °C for 1 h. Human umbilical vein endothelial cells (HUVEC) were purchased from Lonza (Basel, Switzerland) and maintained in EGM2-MV medium in accordance with the supplier's instructions. The cells were starved for 8 h in serum-free MCDB131 supplemented with 0.1% fatty acid-free bovine serum albumin (BSA), and then cultured with medium containing vehicle and increasing amounts of aldosterone (10^{-9}–10^{-7} M), or aldosterone (10^{-8} M) combined with 10^{-5} M eplerenone, in the presence or absence of 20 ng/ml VEGF. After trypsinization, the cells were resuspended in the same media and seeded onto 24-well plates covered with Matrigel at a density of 5×10^4 cells/well.

After the cells had been cultured at 37 °C in 5% CO_2 for 4 h, each well was photographed and the lengths of tubular structures that the cells had formed on the surface of the Matrigel were quantified in five different 0.025 mm^2 areas using Image J software. The length was expressed as mm/mm^2. Similarly, tube formation assay was performed in the presence of 10^{-6} M troglitazone or 10^{-5} M GW9662 to evaluate the role of PPAR gamma in endothelial morphogenesis.

2.3. Reverse transcription-mediated polymerase chain reaction (RT-PCR)

HUVEC were starved in MCDB131 supplemented with 0.1% fatty acid-free BSA overnight, and then stimulated with either vehicle, 10^{-8} M aldosterone, or aldosterone combined with 10^{-5} M eplerenone for 24 h. To investigate the effects of PPAR gamma on the expression of VEGFR-2 mRNA, HUVEC were also treated with 10^{-6} M troglitazone or 10^{-5} M GW9662 for 24 h. After the cells had been washed twice with ice-cold phosphate buffered saline (PBS), total RNA was isolated using ISOGEN reagent (Nippon Gene, Tokyo, Japan). First-strand cDNA was synthesized from 1.5 μg of total RNA using Super Script III (Invitrogen, Carlsbad, California). PCR-based semiquantitative analyses of the gene expression levels of VEGFR-2, PPAR gamma, MR, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were performed with LA Taq (TAKARA, Tokyo, Japan) using the following sets of primers and cycles: 5′-ggcctcttctgtaagacactca-3′ and 5′-gctgggaatagtaaagcccttc-3′, 20 cycles for VEGFR-2, 5′-tctctccgtaatggaagacc-3′ and 5′-gcattatgagaccatccccac-3′, 30 cycles for PPAR gamma, 5′-gtggcgtcatgcgcgccattgttaa-3′ and 5′-tcgaagggctggaaacagagcacct-3′, 25 cycles for MR, and 5′-ccccttcattgacctcaactac-3′ and 5′-gctgatgatcttgaggctgttg-3′, 25 cycles for GAPDH. In the preliminary study, each PCR amplification was performed with various cycles (15, 20, 25, 30 and 35 cycles), and then the optimal cycles were selected as described above in each gene amplification to discriminate semiquantitatively the difference of the mRNA expression level in response to stimuli employed in the experiments. The products were electrophoresed in 2% agarose gel and stained with ethidium bromide. The gels were then photographed and densitometric analyses were carried out using Image J. Each experiment was repeated at least three times. The relative expression levels of each gene were expressed as the ratio of the densitometric value to that of GAPDH.

2.4. Immunoblotting

HUVEC were serum-deprived in MCDB131 supplemented with 0.1% fatty acid-free BSA for 8 h and stimulated with either vehicle, 10^{-8} M aldosterone, or aldosterone combined with 10^{-5} M eplerenone for 24 h. The cells were also treated with 10^{-6} M troglitazone or 10^{-5} M GW9662 for 24 h. The cells were then washed twice with PBS and harvested with lysis buffer containing 50 mM Tris–HCl (pH 7.5), 150 mM NaCl, 2 mM EDTA, 1% Triton X-100, 0.5% sodium deoxycholate, and 0.1% SDS. Then 10 μg of protein was applied to each lane and electrophoresis was conducted on 7.5% (for VEGFR-2) or 10% (for PPAR gamma, MR and GAPDH) polyacrylamide gel under reducing conditions, followed by blotting onto polyvinylidene difluoride (PVDF) membranes. For determination of the expression levels of VEGFR-2, PPAR gamma, MR and GAPDH, rabbit anti-VEGFR-2 monoclonal antibody (Cell Signaling), mouse monoclonal antibody against MR (Affinity Bioreagents), and polyclonal antibodies against PPAR gamma and GAPDH (Santa Cruz) were used as primary antibodies. After treatment with secondary antibodies conjugated with alkaline phosphatase, the proteins were visualized by reaction with 5-bromo-4-chloro-3-indolylphosphate/nitro blue tetrazolium (BCIP/NBT). Each experiment was repeated at least three times.

2.5. Mouse Matrigel injection model

The animal experiments were approved by the Institutional Animal Use Committee of Fukui University. *In vivo* angiogenesis assay using the Matrigel injection model in mice was performed as described previously [10,11]. In brief, 500 μl of Matrigel containing either vehicle, 10^{-8} M aldosterone, or aldosterone combined with 10^{-5} M eplerenone in the presence or absence of 100 ng/ml VEGF, was injected subcutaneously near the abdominal midline of male C57BL/6 mice aged 5–7 weeks ($n = 5$ in each group). The mice were sacrificed 5 days later, and the plug was excised and fixed in 4% paraformaldehyde and paraffin-embedded. Tissue specimens were then sectioned and stained with hematoxylin and eosin. The degree of angiogenesis was determined by counting the number of blood vessels with a luminal area formed in the plug in five different 0.025 mm^2 areas. The sections were also immunostained with rabbit anti-von Willebrand factor antibody and an EnVision kit (DAKO).

2.6. Statistical analysis

Measured values were expressed as mean ± SD. Statistical analysis was performed using the Kruskal–Wallis test, and p values of

less than 0.05 were considered significant. The Dunn procedure was used subsequently for comparing the differences between two groups.

3. Results

3.1. Inhibition of tube formation by aldosterone

The biological effects of aldosterone on endothelial morphogenesis were evaluated in an *in vitro* tube formation assay. HUVEC basically formed tube-like structures on the Matrigel without any stimulation (Fig. 1A and Ba). Addition of aldosterone at 10^{-9}–10^{-7} M inhibited tube formation in a dose-dependent manner (Fig. 1A), the maximal response being achieved at 10^{-7} M. These findings suggested that aldosterone is capable of inhibiting endothelial morphogenesis *in vitro*. The inhibitory effect of aldosterone (10^{-8} M) (Fig. 1Bb and Ca) was reversed to the basal level in the presence of 10^{-5} M eplerenone (Fig. 1Bc and Ca). VEGF at 20 ng/ml enhanced tube formation up to 1.3-fold relative to the basal level (Fig. 1Bd and Cb). Notably, addition of aldosterone at 10^{-8} M exerted inhibitory effects on this VEGF-induced increase in tube formation (Fig. 1Be and Cb). In the presence of eplerenone at 10^{-5} M, the inhibition of VEGF-induced tube formation by aldosterone was markedly reversed almost to the level induced by VEGF alone (Fig. 1Bf and Cb). These observations demonstrate that aldosterone inhibits endothelial morphogenesis *in vitro* through a MR-dependent mechanism.

3.2. Effects of aldosterone on the expression of PPAR gamma, VEGFR-2 and MR

To analyze the effects of aldosterone on the levels of expression of PPAR gamma, VEGFR-2 and MR, we performed reverse transcription mediated PCR and immunoblotting. Semiquantitative PCR-based analyses revealed that the expression of mRNA for PPAR gamma in HUVEC was suppressed to about 40% of the control level by 10^{-8} M aldosterone, and that inhibition was reversed in the presence of 10^{-5} M eplerenone (Fig. 2Aa). Expression of VEGFR-2 mRNA was also found to be downregulated by 10^{-8} M aldosterone to about 70% of the control level, in a manner that was sensitive to eplerenone (Fig. 2Ab). On the other hand, the expression of mRNA for MR was not affected by aldosterone (Fig. 2Ac). We also performed immunoblotting to further confirm the inhibitory effects of aldosterone on the expression of PPAR gamma and VEGFR-2. As shown in Fig. 2B, treatment with 10^{-8} M aldosterone decreased the protein expression of both PPAR gamma and VEGFR-2 in HUVEC (Fig. 2Ba,b), but did not alter the protein expression of MR and GAPDH (Fig. 2Bc,d). Addition of 10^{-5} M eplerenone in turn diminished the aldosterone-induced inhibition of PPAR gamma and VEGFR-2 expression (Fig. 2Ba,b). These data indicate that aldosterone negatively regulates the expression of both PPAR gamma and VEGFR-2 *via* the MR.

3.3. Involvement of PPAR gamma in tube formation and VEGFR-2 expression

To analyze the involvement of PPAR gamma in endothelial morphogenesis, we carried out a tube formation assay using its specific agonist, troglitazone, and an antagonist, GW9662. Stimulation of HUVEC with 10^{-6} M troglitazone enhanced tube formation approximately 1.4-fold relative to the control, as shown in Fig. 3Aa,b and Ba. Troglitazone also stimulated VEGF-induced tube formation to 1.3-fold, as shown in Fig. 3Ad,e and Bb. On the other hand, the selective PPAR gamma antagonist, GW9662, at 10^{-5} M inhibited endothelial morphogenesis to about 80% of the control (Fig. 3Ac and Ba). Moreover, GW9662 inhibited VEGF-induced tube formation to the control level (Fig. 3Af and Bb). Semiquantitative PCR analysis revealed that PPAR gamma activation by troglitazone resulted in the enhancement of VEGFR-2 mRNA expression, while the inactivation by GW9662 reduced the expression of VEGFR-2 mRNA (Fig. 3C). The role of PPAR gamma in the expression of VEGFR-2 in HUVEC was also investigated by immunoblotting (Fig. 3D). Treatment of the cells with 10^{-6} M troglitazone augmented the expression level of VEGFR-2 by approximately 30%, as assessed by densitometric analysis, whereas GW9662 at 10^{-5} M attenuated the expression of VEGFR-2 by about 60% relative to the control level (Fig. 3Da). These data suggested that activation of PPAR gamma mediates the angiogenic morphogenesis of HUVEC *via* upregulation of VEGFR-2 expression.

3.4. MR-mediated inhibition of angiogenesis in vivo

We evaluated the effect of aldosterone on angiogenesis *in vivo* using a Matrigel injection model in mice. The number of cells and blood vessels that infiltrated the Matrigel plug containing vehicle alone were relatively low, as shown in Fig. 4Aa.

Addition of VEGF at 100 ng/ml markedly enhanced the numbers of cells and blood vessels that invaded the plug (Fig. 4Ab). It was noteworthy that 10^{-8} M aldosterone exerted an inhibitory effect on VEGF-induced angiogenesis (Fig. 4Ac), which was clearly cancelled in the presence of eplerenone (Fig. 4Ad and B). Almost all of the cells in the plug were found to be immunopositive for von Willebrand factor (Fig. 4Ae,f). These results demonstrate that aldosterone inhibits angiogenesis through the MR *in vivo*.

4. Discussion

Aldosterone has been reported to mediate various bioactivities in the pathogenesis of cardiovascular diseases, including myocardial hypertrophy, cardiac fibrosis, and endothelial dysfunction [12,13,15]. In the present study, we have shown that aldosterone inhibits endothelial morphogenesis *in vitro* and angiogenesis *in vivo*, and that the angiostatic effects of aldosterone are mediated by downregulation of VEGFR-2 expression in vascular endothelial cells subsequent to attenuation of PPAR gamma expression. This is the first study to have demonstrated directly the inhibitory effects of aldosterone on angiogenesis.

Previous studies have shown that endothelial morphogenesis and angiogenesis are regulated by the MR. Spironolactone, a mineralocorticoid receptor antagonist, appears to reduce neovascularization in fibrin gel implanted in rats [16], although the proangiogenic properties of aldosterone were not observed. In a murine model of hindlimb ischemia, aldosterone has been reported to enhance neovascularization through angiotensin II signaling [17]. On the other hand, it has been suggested that mineralocorticoid blockade enhances angiogenesis in experimental myocardial ischemia [18] and in a brain ischemia model [19]. Therefore, it still remains largely undetermined whether its effect on angiogenesis is stimulatory or inhibitory. Since angiogenesis involves complex biological processes, such as regulation of the expression of receptors for angiogenic growth factors and cytokines in endothelial cells and release of these actors from surrounding tissues in response to various stimuli, the net result of angiogenesis may depend on the cumulative effects of these responses and may vary according to the experimental settings employed. In the present study, we demonstrated that aldosterone inhibited endothelial morphogenesis in an *in vitro* tube formation assay and reduced the expression of VEGFR-2, indicating that aldosterone itself at least attenuates VEGF-dependent angiogenesis in HUVEC upon activation *via* the MR.

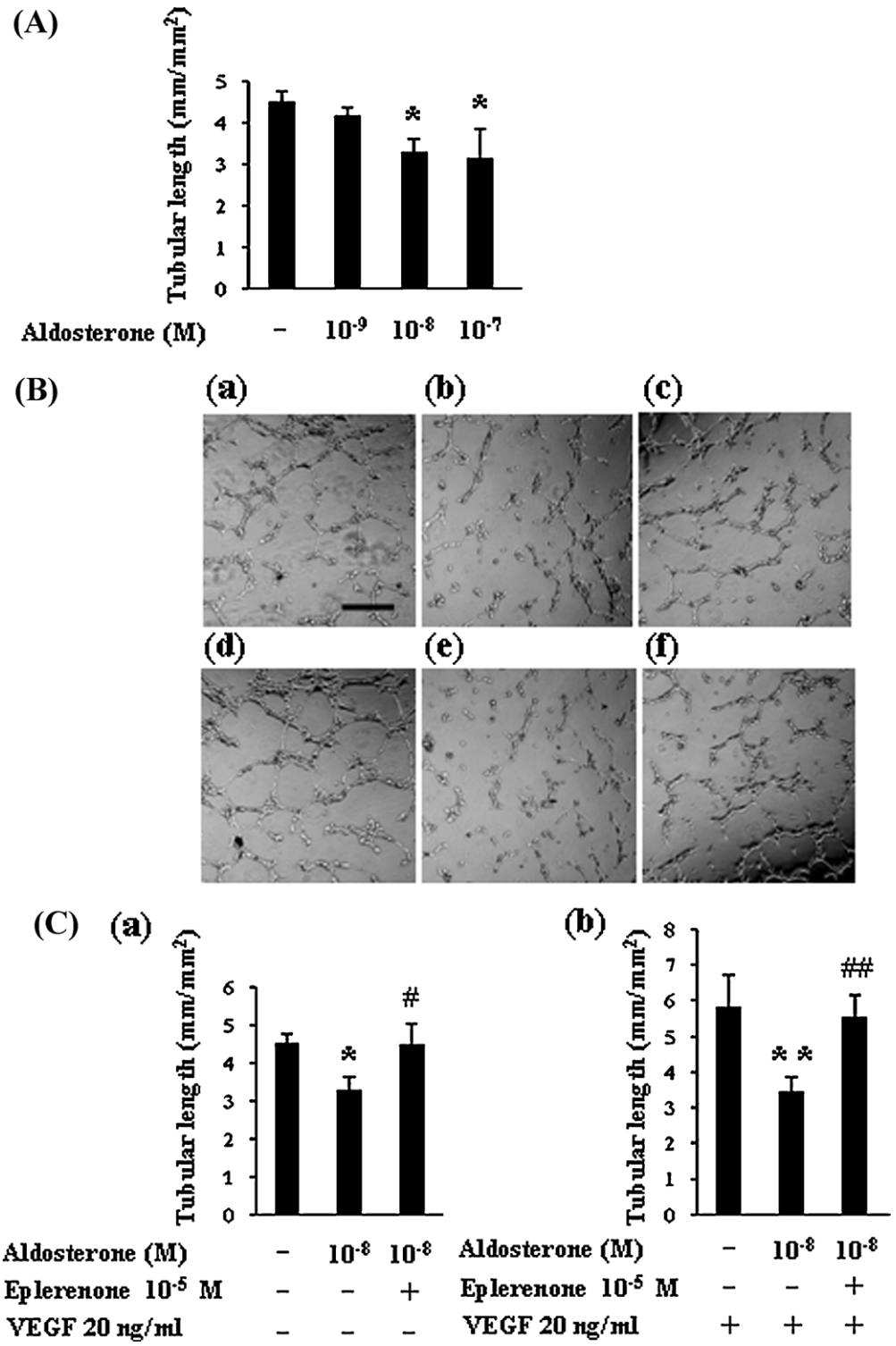

Fig. 1. Aldosterone inhibits endothelial morphogenesis in HUVEC on Matrigel. (A) Aldosterone inhibited tube formation in a dose-dependent manner. Significant inhibitory effects were observed at 10^{-8} and 10^{-7} M. (B) Representative photographs: vehicle alone (a); aldosterone (10^{-8} M) (b); aldosterone (10^{-8} M) and eplerenone (10^{-5} M) (c); VEGF (20 ng/ml) (d); VEGF (20 ng/ml) and aldosterone (10^{-8} M) (e); VEGF (20 ng/ml), aldosterone (10^{-8} M) and eplerenone (10^{-5} M) (f); bar, 500 μm. (C) Quantitative analysis: HUVEC were photographed and the length of tube-like structures was measured in five different 0.25 mm^2 areas using Image J. Inhibition of tube formation by aldosterone (a) and its inhibitory effects on VEGF-induced tube formation (b). $^{*}p < 0.05$ as compared with control; $^{\#}p < 0.05$ as compared with aldosterone; $^{**}p < 0.05$ as compared with VEGF; $^{\#\#}p < 0.05$ as compared with VEGF and aldosterone.

Details of the mechanisms involved in aldosterone-mediated downregulation of gene expression remain unclear. In bone marrow-derived progenitor cells, aldosterone has been shown to inhibit the expression of VEGFR-2 [20]. In that study, it was speculated that the mechanism of inhibition might be mediated by the p38-MAPK pathway. It has also been reported that C-reactive protein and 17β-estradiol downregulate the expression of VEGFR-2 [21,22]. In the former case, it was speculated that nuclear factor-κB activation or reduction of nitric oxide production might be responsible for the downregulation, and in the

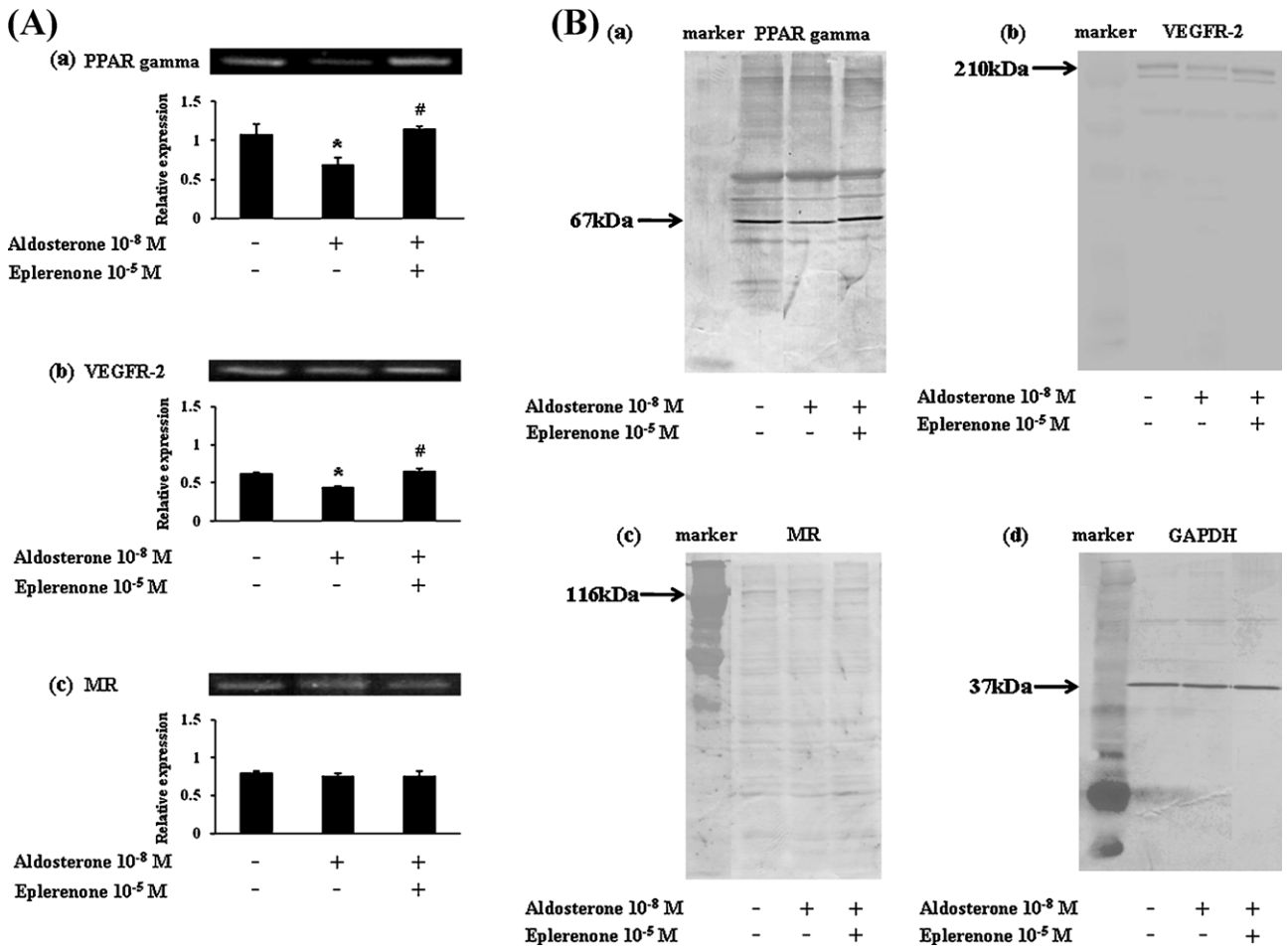

Fig. 2. Aldosterone inhibits the levels of expression of PPAR gamma and VEGFR-2. (A) Effects of aldosterone on expression of mRNAs for PPAR gamma and VEGFR-2. HUVEC were treated with vehicle, aldosterone (10^{-8} M), or a combination of aldosterone (10^{-8} M) and eplerenone (10^{-5} M). Total RNA was extracted from the cells and subjected to semiquantitative RT-PCR. PPAR gamma and VEGFR-2 mRNA expressions were attenuated by aldosterone in a manner sensitive to eplerenon (a and b), while MR expression was not affected by aldosterone (c). $^{*}p < 0.05$ as compared with control, $^{\#}p < 0.05$ as compared with aldosterone. (B) HUVEC were treated similarly to (A), and then 10 μg of protein from cell lysates was subjected to Western analysis (a–d). Note that aldosterone attenuated the expression of PPAR gamma and VEGFR-2 at both the mRNA and the protein level.

latter, estrogen receptor ER alpha/Sp1 and ER alpha/Sp3 complex were demonstrated to interfere with transcription of the VEGFR-2 gene. These types of transcriptional regulation may account for the aldosterone-induced attenuation expression of VEGFR-2 and PPAR gamma in the present study, and this is an issue that awaits further investigation. Taken together with the observation that the PPAR gamma agonist, troglitazone, enhanced both tube formation and the expression of VEGFR-2, which in turn were diminished by the antagonist, GW9662, the present data suggest that aldosterone-evoked downregulation of VEGFR-2 is, at least in part, subsequent to the attenuation of PPAR gamma expression.

Studies of the roles of PPAR gamma in angiogenesis have also yielded variety of results. Although several studies have demonstrated that PPAR gamma agonists enhance the proliferation of endothelial cells and angiogenesis [23], others have yielded contrary findings [24]. Fukunaga et al. reported that stimulation of PPAR gamma with troglitazone or pioglitazone enhanced DNA synthesis in endothelial cells at lower concentrations, whereas higher doses of agonists suppressed endothelial [^{3}H]-thymidine incorporation [25]. In the present study, we also observed that troglitazone stimulated tube formation at 10^{-6} M, but diminished it at 10^{-5} M (data not shown), leading us to speculate that troglitazone exerts an angiogenic effect at relatively low concentrations, and cytotoxic effects at higher concentrations. PPAR gamma has been reported to modulate Sp1 and Sp3 binding on the promoter region of VEGFR-2 gene and then the expression of the mRNA expression [26]. Although these mechanisms might be responsible for the upregulation of VEGFR-2 expression upon PPAR gamma activation observed in our study, precise details of the signal transduction mechanisms downstream of PPAR gamma that regulate the expression of VEGFR-2 and other growth factors await further study.

Aldosterone has been reported to affect the level of expression of PPAR gamma in 3T3-L1 cells. In the differentiation of 3T3-L1 preadipocytes, MR activation has been shown to increase the expression of mRNA for PPAR gamma, accompanied by an increase in the expression of adiponectin [27]. In contrast, it has been shown that aldosterone decreases the expression of PPAR gamma, leading to suppression of adiponectin induction, and that MR antagonism by eplerenone enhances the expression of both PPAR gamma and adiponectin in adipose tissue of obese diabetic mice [28]. In line with that report, our present study demonstrated that MR activation attenuated the expression of PPAR gamma whereas MR blockade reversed, or even enhanced it, possibly resulting in an increase of adiponectin, and a decrease of proinflammatory cytokines such as monocyte chemoattractant protein-1 (MCP-1) and tumor necrosis factor-α (TNF-α). Although the existence of MR responsive element in the promoter region of PPAR gamma gene has not been shown so far, it is possible that MR may modify the

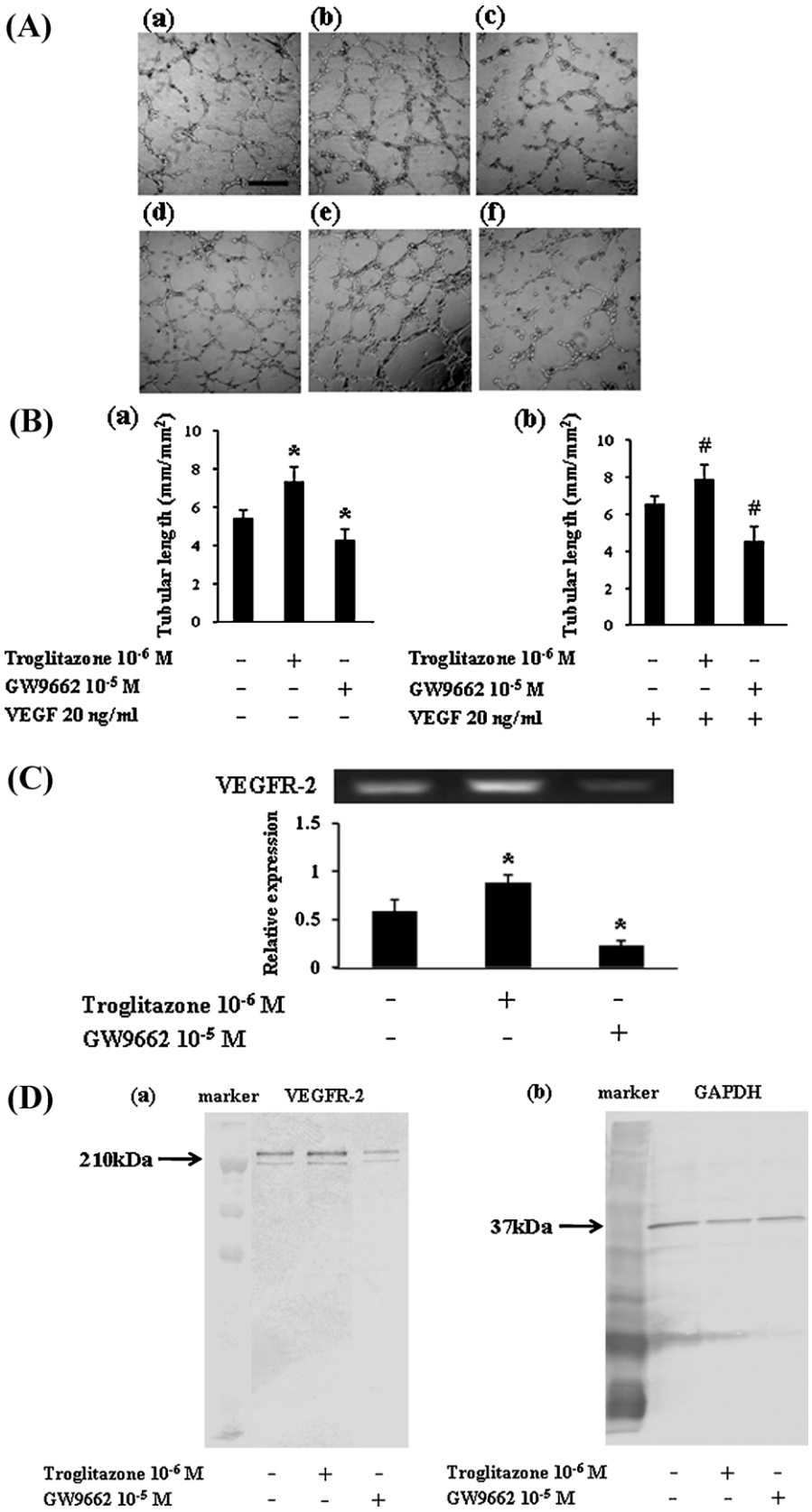

Fig. 3. Involvement of the PPAR gamma pathway in endothelial morphogenesis. (A) Representative photographs: non-treated control (a); troglitazone (10^{-6} M) (b); GW9662 (10^{-5} M) (c); VEGF (20 ng/ml) (d); VEGF (20 ng/ml) and troglitazone (10^{-6} M) (e); VEGF (20 ng/ml) and GW9662 (10^{-5} M) (f); bar, 500 μm. (B) Quantitative analysis of tube formation. Measurements of tube length were performed as in Fig. 1. PPAR gamma activation with troglitazone enhanced tube formation and its inhibition resulted in reduction of tube formation (a); troglitazone enhanced VEGF-induced tube formation and GW9662 inhibited VEGF-induced tube formation (b); bar, 500 μm. $^{*}p < 0.05$ as compared with non-treated control; $^{\#}p < 0.05$ as compared with VEGF. (C) Effects of PPAR gamma activity on VEGFR-2 mRNA expression. HUVEC were treated with vehicle alone, troglitazone (10^{-6} M), and GW9662 (10^{-5} M) and 1.5 μg of total RNA from cell lysates was subjected to RT-PCR

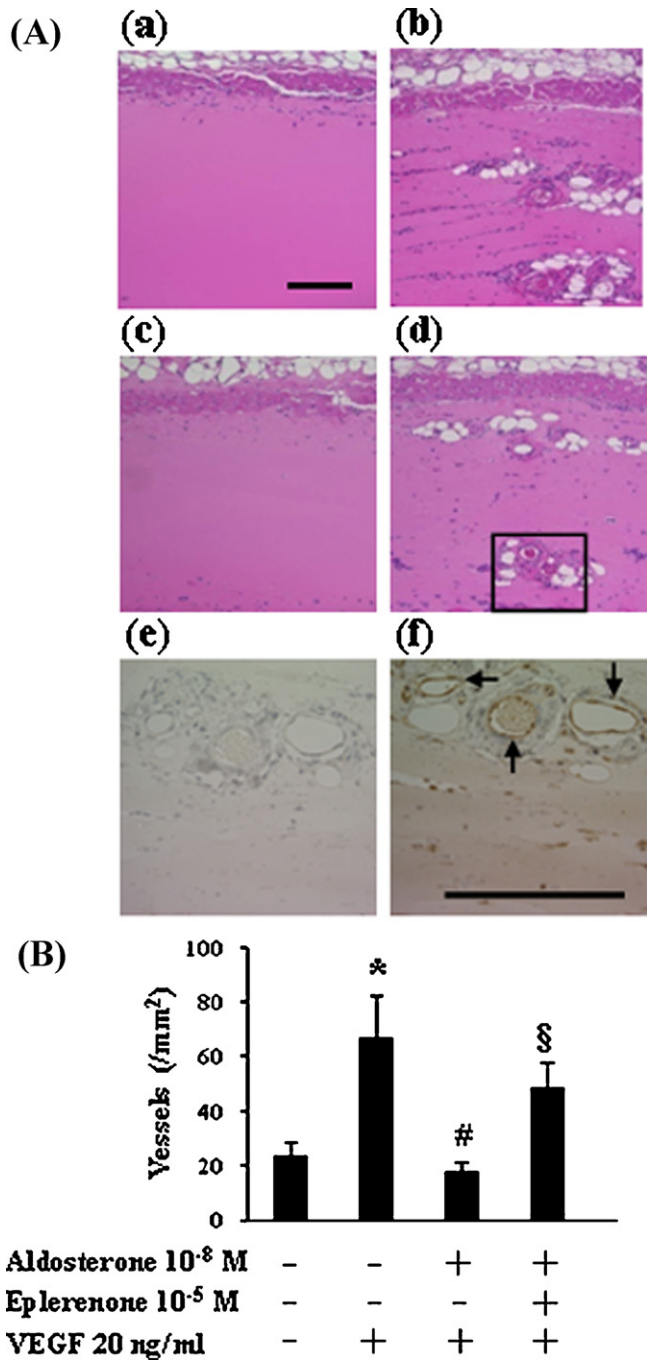

Fig. 4. Aldosterone inhibits angiogenesis *in vivo* in a mouse Matrigel injection model. (A) Matrigel plug containing vehicle alone (a); VEGF (100 ng/ml) (b); VEGF and aldosterone (10^{-8} M) (c); and VEGF, aldosterone and eplerenone (10^{-5} M) (d); sections were stained with hematoxylin–eosin (a–d), and immunostained using non-immune control IgG (e) and anti-von Willebrand factor antibody (f); bar, 200 μm. Inset in Ad is the same area demonstrated in Ae,f. Arrows indicate the vessels formed in the Matrigel plug. Note that the vessels are surrounded by von Willebrand factor-positive endothelial cells. (B) Quantitative analysis of angiogenesis in the Matrigel plug. The number of vessels was counted in five different areas in 1 mm^2. $^{*}p < 0.05$ compared with control, $^{\#}p < 0.05$ compared with VEGF (100 ng/ml), $^{\S}p < 0.05$ compared with aldosterone (10^{-8} M) and VEGF (100 ng/ml).

affinity of the DNA binding protein such as Sp1, Sp3 and Ap1, and regulate the expression level of PPAR gamma. These possibilities should also be elucidated in the future study.

In conclusion, we have found that aldosterone inhibits angiogenesis *in vitro* and *in vivo* through downregulation of VEGFR-2 subsequent to reduction of PPAR gamma expression, and that MR blockade by eplerenone reverses this aldosterone-induced antiangiogenic effect and VEGFR-2 downregulation. The present study may provide the new insight into the possible therapeutic application of MR blockade for cardiovascular diseases.

Acknowledgments

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analysis as described in Materials and methods. VEGFR-2 mRNA expression was stimulated by troglitazone and inhibited by GW9662, significantly. $^{*}p < 0.05$ as compared with control. (D) Effects of PPAR gamma activity on VEGFR-2 protein expression. HUVEC were treated with vehicle alone, troglitazone (10^{-6} M), and GW9662 (10^{-5} M) and 10 μg of protein from cell lysates was subjected to Western analysis. VEGFR-2 expression was stimulated by troglitazone and inhibited by GW9662 at protein level.

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