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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

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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

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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