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Aldosterone increases osteopontin gene expression in rat endothelial cells

Toru Sugiyama, Takanobu Yoshimoto *, Yuki Hirono, Noriko Suzuki, Maya Sakurada, Kyoichiro Tsuchiya, Isao Minami, Fumiko Iwashima, Haruna Sakai, Toru Tateno, Ryuji Sato, Yukio Hirata

Department of Clinical and Molecular Endocrinology, Tokyo Medical and Dental University Graduate School, Tokyo 113-8519, Japan

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Abstract

Aldosterone is currently recognized as one of the important risk hormones for cardiovascular disease. However, the cellular mechanism by which aldosterone affects the process of cardiovascular injury has not been well understood. In the present study, we investigated whether aldosterone induces pro-inflammatory genes expression in rat aortic endothelial cells. Aldosterone significantly increased steady-state osteopontin mRNA and protein levels, but not those of adhesion molecules or chemokine. The stimulatory effect of aldosterone on osteopontin expression was time-dependent (3–24 h) and dose-dependent (10^{-10}–10^{-6} M), and abolished by a mineralocorticoid receptor (MR) antagonist spironolactone, but not by a glucocorticoid receptor antagonist RU486. The aldosterone-induced osteopontin mRNA expression was completely blocked by a transcription inhibitor, actinomycin D, and a protein synthesis inhibitor, cycloheximide. Thus, the present study demonstrated for the first time that aldosterone directly acts on endothelial cells to induce osteopontin gene expression via MR-mediated genomic action, which may be responsible for the initiation of inflammation and fibrosis in cardiovascular tissue induced by aldosterone.

Keywords: Aldosterone; Osteopontin; Aldosterone-induced vasculitis; Endothelial cells

It has been well recognized that endothelial injury is a pivotal event in the pathogenesis of cardiovascular disease [1,2]. It has been shown that endothelial dysfunction is an early event predisposing atherosclerotic and hypertensive vascular diseases [3]. Under pathological conditions, pro-inflammatory mediators, such as cytokines, Ang II, prostaglandins, and reactive oxygen species, activate endothelial cells to induce a series of gene expression, including adhesion molecules, chemokines, and extracellular matrix, all of which render leukocyte extravasation from lumen to vessel wall, thereby leading to the development of vascular diseases through an inflammatory process.

Osteopontin is a multifunctional glycophosphoprotein secreted by many cell types, including vascular smooth muscle cells and endothelial cells [4]. Osteopontin has arginine–glycine–aspartic acid (RGD) cell-binding sequence and interacts with integrin receptors ($\alpha v\beta 3$, $\alpha v\beta 5$, and $\alpha v\beta 1$) and CD44 receptors (v6 and/or v7) in a RGD-dependent manner as a chemoattractant factor for various cell types, notably monocytes/macrophages [5,6]. In addition, osteopontin also interacts with fibronectin and collagen, suggesting its possible role in matrix organization and stability [7]. It has been reported that osteopontin mRNA and protein are abundantly expressed in the atherosclerotic vascular lesions, such as neointima and calcified atheromatous plaques [8]. These findings suggest that osteopontin serves as a critical mediator during the pro-inflammatory and

* Corresponding author. Fax: +81 3 5803 0172.
E-mail address: tyoshimoto.cme@tmd.ac.jp (T. Yoshimoto).

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pro-fibrotic process, thereby contributing to the development and/or progression of cardiovascular diseases.

A series of recent clinical studies using mineralocorticoid receptor (MR) antagonists have demonstrated the pathophysiological importance of aldosterone in the development of cardiovascular disease as a risk hormone [9,10]. Classically, cardiovascular effect of aldosterone has been thought to be mediated via its volume expansion/hypertensive effect. However, numerous experimental studies have postulated the possible direct cardiovascular effects by aldosterone as responsible for the initiation of cardiovascular inflammation and fibrosis (''aldosterone-induced vasculitis'') [11–13], although the cellular and molecular mechanism(s) by which aldosterone induces such cardiovascular injury remains unknown.

The present study was undertaken to explore whether aldosterone directly induces the expression of a series of pro-inflammatory genes in cultured rat aortic endothelial cells, and further elucidate the cellular mechanism by which aldosterone induces osteopontin gene expression.

Materials and methods

Materials. Aldosterone was purchased from Acros Organics (Geel, Belgium), spironolactone from Sigma (St. Louis, MO), and actinomycin D, cycloheximide, and RU486 from Biomol Research Laboratories (Plymouth, PA). PCR primers were synthesized by JbioS (Saitama, Japan).

Cell culture. Rat aortic ECs (RAECs) prepared by the explant method [14] were cultured in Medium 199 containing 10% fetal bovine serum (Cell Culture Laboratories, Cleveland, OH) and 30 μg/ml endothelial cell growth supplement (BD Biosciences, Bedford, MA) on collagen-coated dish (IWAKI, Chiba, Japan) at 37 °C in an atmosphere of 5% CO_2 [15]. The RAECs used in the present study showed typical cobblestone appearance and more than 95% of cells incorporated dil-acetylated LDL with negative immunostaining for α-smooth muscle actin [15]. Subcultured RAECs (4–10th passages) grown to confluence, were starved with Medium199 containing 0.5% calf serum for 24 h and used for subsequent experiments.

Quantification of mRNA. Rat osteopontin, ICAM-1, VCAM-1, and MCP-1 mRNA levels were quantified with real-time RT-PCR using fluorescent SYBR green technology (LightCycler: Roche Molecular Biochemicals, Mannheim, Germany), as described previously [15]. Rat MCP-1 and acid ribosomal phosphoprotein P0 (ARPP P0), a housekeeping gene, mRNA levels were quantitated by TaqMan fluorescence methods as described [15], except for the use of QuantiTect Probe PCR kit (Qiagen) and LightCycler technology. Total RNA was extracted, first-strand cDNA was synthesized, and the amplification reaction was performed as described previously [15]. The sequences of PCR primers and TaqMan probe were as follows: osteopontin (forward: 5′-AGTGG TTTGCCTTTGCCTGTT-3′, reverse: 5′-TCAGCCAAGTGGCTAC AGCAT-3′; product size 122-bp); ICAM-1 (forward 5′-AGCTCTTC AAGCTGAGCGACAT-3′, reverse 5′-ACTCGCTCTGGGAACGA ATACA-3′; product size 118 bp); VCAM-1 (forward 5′-GCGAAGG AAACTGGAGAAGACA-3′, reverse 5′-ACACATTAGGGACCGT GCAGTT-3′; product size 128 bp); MCP-1 (forward 5′-TCTCTTCC TCCACCACTATGCA-3′, reverse 5′-GGCTGAGACAGCACGTGG AT-3′, TaqMan probe: 5′-TCACGCTTCTGGGCCTGTTGTTCA-3′; product size 91 bp); ARPP P0 (forward: 5′-TAGAGGGTGTCCG CAATGTG-3′, reverse: 5′-GACAAAGCCAGGACCCTTTTGT-3′, TaqMan probe: 5′-ACCCGACTGTTGCCTCAGTGCCTCACTC CA-3′; product size 107-bp). In both the SYBR green and TaqMan real-time PCR methods, the fluorescence data were quantitatively analyzed using serial dilution of control samples included in each reaction to produce a standard curve. To compare the relative expression of each gene, ARPP P0 was used as an endogenous internal control; the relative levels of each mRNA to that of ARPP P0 were calculated and shown in each figure.

Enzyme-linked immunosorbent assay (ELISA). Confluent RAECs in 6 cm collagen-coated plates were incubated with aldosterone for 24 h; concentrations of osteopontin in medium were determined by commercially available ELISA kit (Immuno-Biological Laboratories, Gunma, Japan) according to the manufacturer's instructions.

Statistical analysis. Data were expressed as means ± SEM. Differences between groups were examined for statistical significance using unpaired *t* test or ANOVA with Dunn's post hoc test, if they were appropriate. *P* values less than 0.05 were considered statistically significant.

Results

Aldosterone induces osteopontin expression in endothelial cells

We first examined whether aldosterone directly induces pro-inflammatory genes (ICAM1, VCAM1, MCP-1, and osteopontin) in cultured RAECs. As shown in Fig. 1, aldosterone (10^{-8} M) significantly increased steady-state osteopontin mRNA levels in a time-dependent manner (3–24 h) (Fig. 1A); a significant (about 1.5-fold) increase was observed at 3 h, which peaked at 24 h (4-fold increase). Aldosterone dose-dependently (10^{-10}–10^{-6} M) increased steady-state osteopontin mRNA levels in RAECs (Fig. 2A); about 1.3-fold increase ($p < 0.05$) was induced by as low as 10^{-10} M and a 4-fold increase by 10^{-6} M. Likewise, aldosterone dose-dependently (10^{-10}–10^{-6} M) increased the secretion of osteopontin into media (Fig. 2B); about 1.2-fold

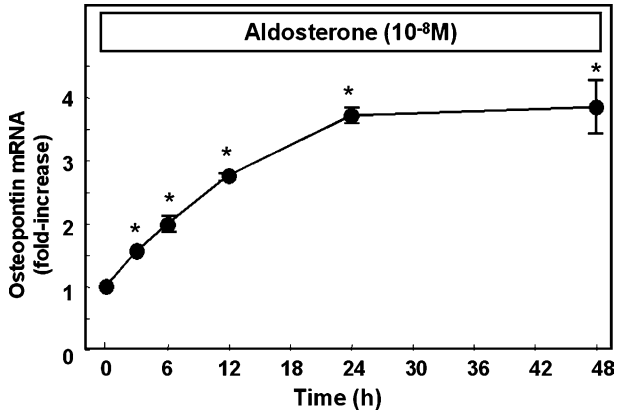

Fig. 1. Aldosterone time-dependently increases osteopontin mRNA expression in rat endothelial cells. RAECs were incubated with aldosterone (10^{-8} M) for the indicated times. Osteopontin mRNA levels were measured by real-time RT-PCR. Each circle shown as fold-increase over the value at 0 h (control) is the mean from five independent experiments; bar shows SE. *$p < 0.05$ vs. control.

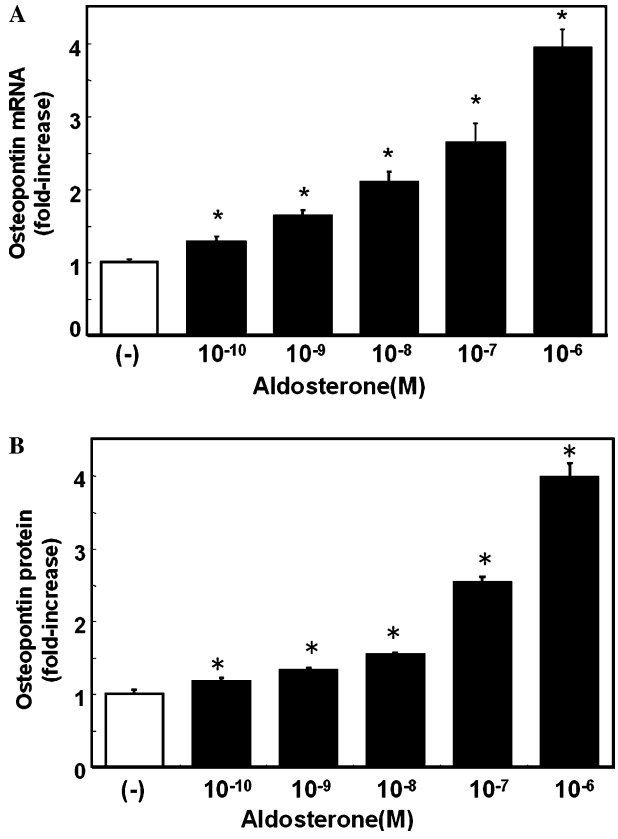

Fig. 2. Aldosterone dose-dependently increases osteopontin mRNA and protein expression in rat endothelial cells. RAECs were incubated with aldosterone in the indicated concentrations for 24 h. (A) Osteopontin mRNA levels were measured by real-time RT-PCR. (B) Concentrations of osteopontin in medium were measured by ELISA. Each column shown as fold-increase over the values without aldosterone (control) is the mean from five independent experiments; bar shows SE. $^{*}p < 0.05$ vs. control.

increase ($p < 0.05$) was induced by as low as 10^{-10} M and about a 4-fold increase by 10^{-6} M. By contrast, steady-state mRNA level of either ICAM1, VCAM1 or MCP-1 did not show any significant change after stimulation with aldosterone (data not shown).

Aldosterone increases osteopontin expression via MR

To determine whether aldosterone-induced osteopontin expression is mediated via MR or glucocorticoid receptor (GR), the effects of a selective MR and GR antagonist were examined. The aldosterone (10^{-8} M)-induced osteopontin gene expression was blocked by pretreatment with a selective MR antagonist, spironolactone (10^{-6} M), but not by a selective GR antagonist, RU486 (10^{-6} M) (Fig. 3A). The aldosterone-stimulated osteopontin secretion was also blocked by spironolactone (Fig. 3B). RAEC used in our study expressed both MR and GR mRNAs as revealed by RT-PCR (data not shown). Therefore, aldosterone-induced osteopontin expression in endothelial cells is mediated exclusively via MR, but not via GR.

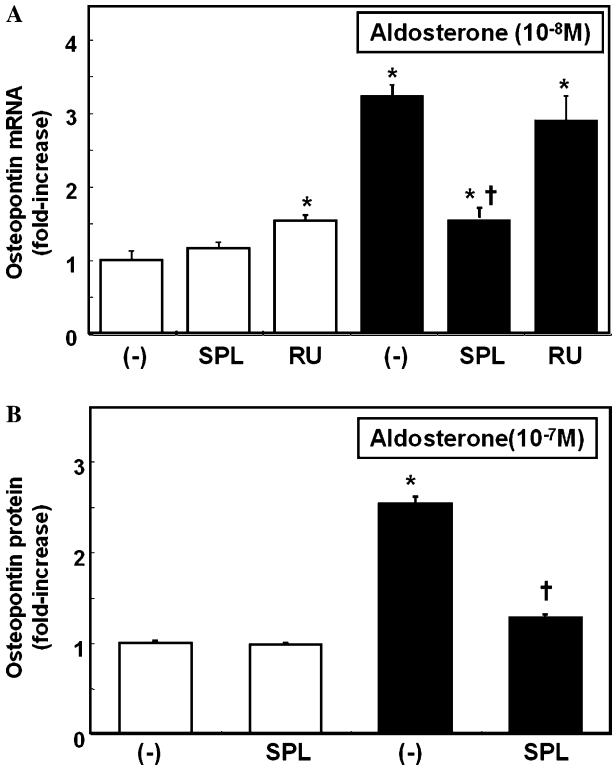

Fig. 3. Effects of spironolactone and RU486 on aldosterone-stimulated osteopontin expression in rat endothelial cells. After pretreatment with or without spironolactone (SPL) (10^{-6} M) or RU486 (10^{-6} M) for 1 h, RAECs were exposed to aldosterone (10^{-8} M) for 24 h; osteopontin mRNA levels and protein concentrations in medium were measured. The data from five independent experiments were calculated and plotted as described in Fig. 2; bar shows SE. $^{*}p < 0.05$ vs. control. $^{\dagger}p < 0.05$ vs. aldosterone alone.

Aldosterone increases osteopontin expression by a genomic action

The aldosterone-induced upregulation of osteopontin mRNA was completely blocked by pretreatment with a transcription inhibitor, actinomycin D (5×10^{-6} M) (Fig. 4A), indicating its transcription-dependent genomic action. To further examine whether aldosterone-induced osteopontin gene expression requires de novo protein synthesis, we next examined the effect of a protein synthesis inhibitor, cycloheximide. The aldosterone-induced osteopontin gene expression was also completely blocked by pretreatment with cycloheximide (10 μg/ml) (Fig. 4B), suggesting the requirement of de novo protein synthesis for its genomic action.

Discussion

The present study demonstrated for the first time that aldosterone directly induced osteopontin gene and protein expression in rat endothelial cells, whose effects were completely blocked by a selective MR receptor antagonist. The aldosterone-induced upregulation of

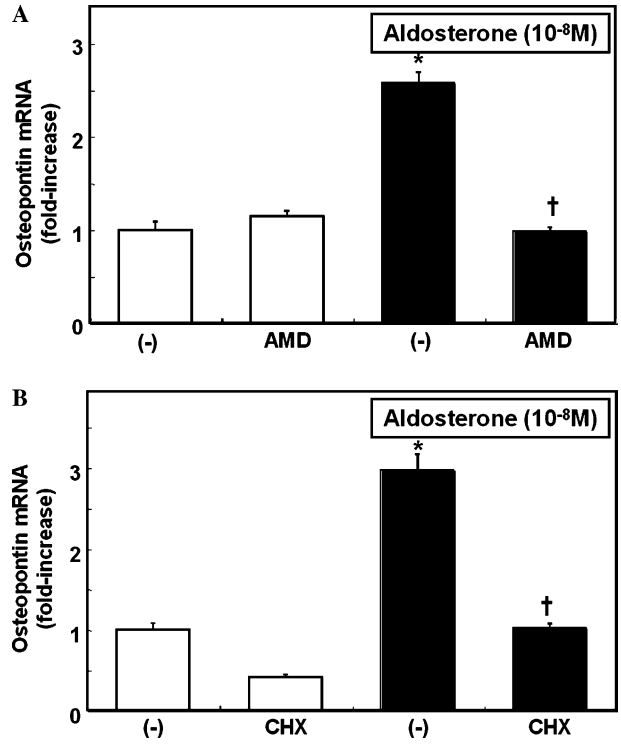

Fig. 4. Effects of actinomycin D and cycloheximide on aldosterone-stimulated osteopontin mRNA expression in rat endothelial cells. After pretreatment (A) with or without actinomycin D (AMD) (5×10^{-6} M) or (B) cycloheximide (10 μg/ml) for 1 h, RAECs were exposed to aldosterone (10^{-8} M) for 24 h; osteopontin mRNA levels were measured. The data from five independent experiments were calculated and plotted as described in Fig. 2; bar shows SE. $^{*}p < 0.05$ vs. control. $^{\dagger}p < 0.05$ vs. aldosterone alone.

osteopontin mRNA was completely blocked by a transcription inhibitor and a protein synthesis inhibitor, suggesting that aldosterone-induced osteopontin expression is mediated by a genomic action with requirement of de novo protein synthesis.

It has been recently recognized that aldosterone plays a pivotal role in the development and/or progression of cardiovascular diseases [9–13]. Accumulating lines of evidence from animal experiments reveal that aldosterone, independent of its blood pressure-elevating effect, induced vascular inflammation (“aldosterone-induced vasculitis”) characterized by infiltrations of mononuclear cells and expression of a variety of pro-inflammatory genes (including osteopontin, ICAM-1, VCAM-1, and MCP-1) in vascular wall [11–13]. It has been shown that pro-inflammatory genes expression in endothelial cells play key roles in the initiation and the development of vascular inflammation [12]. In the present study, however, aldosterone induced solely osteopontin gene expression in RAECs among a variety of pro-inflammatory genes tested. This is in sharp contrast with our previous observation that angiotensin II induced upregulation of many pro-inflammatory genes, including osteopontin, MCP-1, ICAM-1, VCAM-1, PAI-1, and tissue factor, in the same cells [16]. Thus, the modes of action and the mechanisms responsible for initiating vascular inflammation appear to differ between aldosterone and angiotensin II.

It has been reported that MR mRNA is expressed not only in epithelial cells, but also in non-epithelial tissue such as cardiovascular tissue [17,18]. We have previously reported that RAECs expressed MR mRNA [15]. In the present study, the minimum effective concentrations of aldosterone (10^{-10} M) required for osteopontin expression in RAECs appeared to be almost comparable to its physiological and pathophysiological concentrations in the circulation ($\sim 10^{-9}$ M) [19]. Furthermore, the aldosterone-induced osteopontin gene expression in RAECs was inhibited by a selective MR antagonist (spironolactone) at a concentration (10^{-6} M) almost equivalent to those in plasma concentrations (10^{-7}–10^{-6} M) in the clinical setting [20]. These results suggest that the aldosterone in its physiological and/or pathophysiological concentrations regulates osteopontin gene expression via MR in endothelium.

The present study also shows that aldosterone-induced upregulation of osteopontin gene in endothelial cells is completely blocked by both a transcription inhibitor (actinomycin D) and a protein synthesis inhibitor (cycloheximide). It has been reported that osteopontin promoter contains a hormone response element that can be modulated by adrenal steroids [21]. However, our data suggest that aldosterone-induced osteopontin mRNA expression requires de novo protein synthesis. The present finding is analogous to the fact that the aldosterone-induced ENaC expression on apical membrane of renal epithelial cells is partly dependent on the immediate protein synthesis, such as serum and glucocorticoid inducible protein kinase 1 (Sgk1) [22]. It is therefore possible to speculate that aldosterone may initially induce expression of some unidentified protein(s), for example Sgk1, thereby leading to osteopontin gene expression, although its exact molecular mechanism remains to be determined.

Osteopontin, an acidic, calcium-binding, and phosphorylated protein, exists in the extracellular matrix of mineralized tissues and in the circulation [6]. Osteopontin expression in blood vessels has been shown to be induced in response to multiple stimuli, such as Ang II or during vascular repair or regeneration [23,24], and to activate macrophages and T-cells to migrate and produce other cytokines via αvβ3 or CD44 receptors [25,26]. Furthermore, osteopontin has the ability to stimulate proliferation and migration of vascular smooth muscle cells via αvβ3 integrins, thereby leading to vascular remodeling [27,28]. Thus, it could be hypothesized that aldosterone-induced osteopontin expression in endothelial cells plays an initial role in the development of aldosterone-induced vascular inflammation and fibrosis via its cell-mediated immunity and vascular remodeling.

In conclusion, our present study demonstrates that aldosterone directly induces osteopontin expression in endothelial cells via MR-dependent genomic action, which may be responsible for the initiation of "aldosterone-induced vasculitis."

Acknowledgments

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