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ORIGINAL ARTICLE

Aldosterone-induced *osteopontin* gene transcription in vascular smooth muscle cells involves glucocorticoid response element

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Osteopontin (OPN) is known to be one of the cytokines that is involved in the vascular inflammation caused by aldosterone (Aldo). Previous reports have shown that Aldo increases OPN transcripts, and the mechanisms for this remain to be clarified. In this study, we investigated how Aldo increases OPN transcripts in the vascular smooth muscle cells of rats. Aldosterone increased OPN transcripts time-dependently as well as dose-dependently. This increase was diminished by eplerenone, a mineralocorticoid receptor (MR) antagonist. Luciferase promoter assays showed that the *OPN* promoter deleted to the −1599 site retained the same promoting ability as the full-length *OPN* promoter when stimulated by 10^{-7} M Aldo, but the promoter deleted to the −1300 site lost the promoting ability. A glucocorticoid response element (GRE) is located in that deleted region. Luciferase assays of a mutated promoter without the GRE lost the luciferase upregulation, although mutated promoters with the deletion of other consensus sites maintained the promoter activity. The binding of the Aldo–MR complex to the GRE fragment was confirmed by an electrophoretic-mobility shift assay. This is the first report showing that Aldo regulates the transcriptional levels of OPN and inflammatory responses in the vasculature through a specific GRE site in the *OPN* promoter region.
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Keywords: aldosterone; glucocorticoid response element; mineralocorticoid receptor; osteopontin

INTRODUCTION

The renin–angiotensin–aldosterone (Aldo) system is known to be the most pivotal machinery in the maintenance of blood pressure. The final mediator of this system, Aldo, is the major hormone that controls sodium reabsorption. Aldo elevates blood pressure through volume expansion following sodium absorption in the renal distal tubules. Aldo binds to the mineralocorticoid receptor (MR) in the cytoplasm of distal renal tubules. After moving to the nucleus, the Aldo–MR complex binds the glucocorticoid response element (GRE) in the promoter sequences of the epithelial sodium channel gene in order to activate transcription.[1]

Two recent clinical trials, the Randomized Aldactone Evaluation Study and the Eplerenone Post-Acute Myocardial Infarction Heart Failure Efficacy and Survival Study, showed that the MR antagonists, spironolactone and eplerenone (Eplr), improved the prognosis of chronic heart failure patients, even at doses below the threshold that causes significant renal effects.[2,3] This finding suggests that MR antagonism may have a direct protective effect on the cardiovascular system. MR is distributed in not only distal renal tubules but also non-epithelial tissues, such as the cardiovascular system, including vascular smooth muscle cells (VSMCs).[4,5] There is a large body of evidence that Aldo induces inflammatory changes in the vasculature leading to deterioration in vascular function.[6,7]

Aldo and sodium load have been reported to cause inflammation and fibrosis in the cardiovascular system.[8,9] However, because the heart and the vasculature do not have enough expression of 11β-hydroxysteroid dehydrogenase type 2,[10] the precise mechanism of how Aldo binds MR and expresses its pro-inflammatory action without glucocorticoid deactivation has not yet been elucidated.

Osteopontin (OPN), which is an acidic glycoprotein of ~300 amino acids, was first described after it was isolated from bovine bone matrix.[11,12] OPN has been found to be expressed in various cell types in addition to osteocytes.[13,14] This secretory protein was reported to be involved in the metastasis of cancer cells and the inflammation of rheumatoid arthritis through the Arginine–Glycine–Aspartic Acid (RGD) sequence located adjacent to the cleavage site of matrix metalloproteinases.[15,16] OPN levels have been reported to be increased in atherosclerotic lesions.[17,18] Various types of cells have been reported to have increased *OPN* gene expression after Aldo stimulation.[19,20] Furthermore, patients with primary aldosteronism have a higher plasma concentration of OPN than patients with primary hypertension, even when they have the same blood pressure.[21] After considering all of these

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results, we hypothesized that Aldo might cause vascular inflammation and atherosclerosis through the augmentation of OPN synthesis. Thus, we decided to investigate the mechanism of *OPN* gene activation in order to understand the inflammatory effects of Aldo. Although VSMCs are one of the main targets of Aldo in the vasculature, an analysis of the Aldo-induced inflammatory reaction in VSMCs has not yet been performed. Thus, in the current study, we examined how Aldo induced *OPN* gene expression in rat VSMCs (rVSMCs).

METHODS

Materials

Monoclonal antibody against OPN was purchased from Immuno-Biology Laboratories (Gunma, Japan). GR and MR antibody were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Aldosterone was purchased from Toronto Research Chemicals (Ontario, Canada). Eplr was a generous gift from Pfizer. Chemical reagents were purchased from Wako Pure Chemicals (Osaka, Japan).

Cell culture

Rat VSMCs were cultured from rat thoracic aortas following the explant method as previously described.[22] The cells migrating from male Wistar rat (8-week-old) aortas fragments were subcultured and expanded in Dulbecco's modied Eagle's medium with 10% fetal bovine serum, 100 U ml^{-1} penicillin and 100 μg ml^{-1} streptomycin. All experiments utilized VSMCs at passage 6 or less. For serum-free experiments, VSMCs were cultured for 48 h in the media containing 1% fetal bovine serum before the experiment. In order to evaluate the inhibition ability of Eplr on the *OPN* gene transcription, rVSMCs were stimulated by 10^{-7} M Aldo after pretreatment with 10^{-5} M Eplr for 1 h.

OPN gene transcription activation by Aldo in rVSMCs

Rat VSMCs were stimulated with various concentrations of Aldo (10^{-10}, 10^{-9}, 10^{-8}, 10^{-7} and 10^{-6} M) for 24 h. In order to assess the time-dependency, rVSMCs were stimulated with 10^{-6} M Aldo for 1.5, 3, 6, 12 or 24 h. After washing with phosphate-buffered saline and scraping, total RNA was precipitated using a Fastpure RNA Kit (Takara Bio Inc., Otsu, Japan).

Real-time reverse transcription-PCR

The primers used for quantitative real-time reverse transcription-PCR (OPN, glyceraldehyde-3-phosphate dehydrogenase) were purchased from Takara. The experiments were performed using SYBR One-Step qRT-PCR kits (Invitrogen, Carlsbad, CA, USA) and PRISM 7000 sequence detection system (Applied Biosystems, Carlsbad, CA, USA) according to the manufacturer's instructions, with glyceraldehyde-3-phosphate dehydrogenase as the internal control. The primers used were as follows:

OPN
Forward, 5′-CCAGCACACAAGCAGACGTT-3′;
Reverse, 5′-TCAGTCCATAAGCCAAGCTATCAC-3′
Glyceraldehyde-3-phosphate dehydrogenase
Forward, 5′-TCCACCACCCTGTTGCTGTA-3′;
Reverse, 5′-ACCACAGTCCATGCCATCCAC-3′.

The data were analyzed by ABI PRISM 7000 SDS software version 1 (Applied Biosystems).

Construction of the *OPN* gene promoters

The whole sequence of the *OPN* gene promoter (2284 base pairs: GenBank AF017274) was cloned from rat genomic DNA using KOD FX DNA polymerase (Toyobo Co., Ltd., Osaka, Japan). The primers used were as follows:

Forward, 5′-GGATGTCCTTCTCTGCTTTGCAGAACT-3′;
Reverse, 5′-AGTCTCCTGCGGCAAGCATTCTC-3′.

This promoter sequence segment was ligated upstream of the firefly luciferase gene in the pGL3-Basic Vector (Promega, Madison, WI, USA). By using this plasmid as a template, four plasmids with deleted sequences of the *OPN* gene promoter were constructed. Applied primers were as follows:

−1599 (5′-CTTAGAATGCATTCACCAAGAGATAACCCGA-3′),
−1300 (5′-CCCATTTGAATACCTCTGAACTATTCAGTAACG-3′),
−795 (5′-GTTTAGATAGCGCGAGAACCATCACC-3′),
−536 (5′-GCCTCAAACTCACGGTGATCTTTT-3′).

These plasmids were subjected to direct sequencing from the 5′ side and the 3′ side with RV primer 3 and GL primer 2, respectively. Plasmid DNAs were transfected to rVSMCs using TransIT-LT1 (Mirus Bio LLC, Madison, WI, USA). Promoter activity was determined by a GloMax 96 Microplate Luminometer (Promega) using a Dual-Glo Luciferase Assay System (Promega). As for the concentration of Aldo 10^{-7} M, in the promoter assay, we referred other investigators' studies[23,24] and our results obtained in the section of *OPN* gene transcription activation (Figure 1).

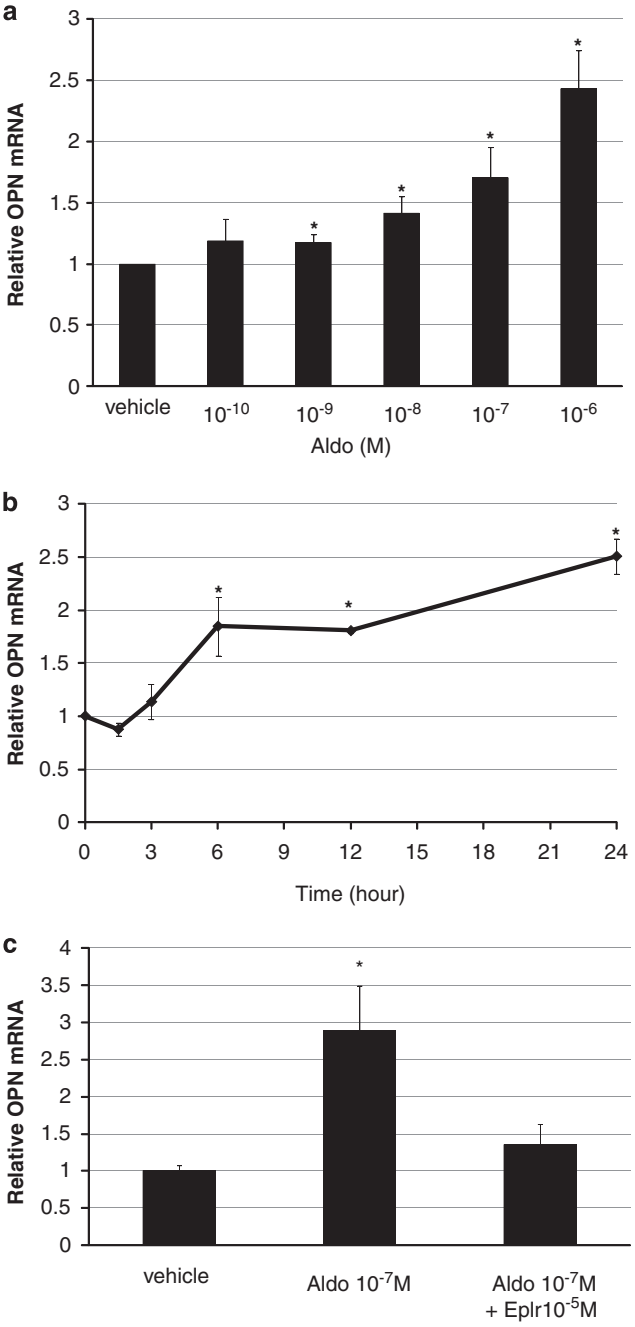

Figure 1 *Osteopontin* (*OPN*) gene transcription induced by aldosterone (Aldo). (**a**) Concentration-dependency. *$P<0.05$ compared with vehicle. (**b**) Time-dependency. *$P<0.05$ compared with control. (**c**) Aldo-induced *OPN* gene transcription was inhibited by Eplr. *$P<0.05$ compared with vehicle.

Construction of plasmid DNAs containing transcription factor binding site-defective promoters

Primers with a defective sequence for CREB (cAMP response element-binding, from −1460 to −1453), GRE (from −1404 to −1386) and the GRE consensus sequence only (from −1394 to −1386), which were present in the target range, were designed and constructed using the primer sets below and QuikChange Site-Directed Mutagenesis Kits (Agilent Technologies, Santa Clara, CA, USA) according to the manufacturer's instructions.

CREB defective
Sense: 5′-GGCTAGTACAACAAAGGTTTTCTAATCTCAATTACTGC-3′
Antisense: 5′-GCAGTAATTGAGAGATTAGAAAACCTTTGTTGTACTAGCC-3′
GRE defective
Sense: 5′-CTGGGGATGTAAGGTATTTCAGGTAATGGAAG-3′
Antisense: 5′-CTTCCATTACCTGAAATACCTTACATCCCCAG-3′
GRE consensus defective
Sense: 5′-GATGTAAGGTAATAAGTCCTATTTCAGGTAATGGAAGAAAG-3′
Antisense: 5′-CTTTCTTCCATTACCTGAAATAGGACTTATTACCTTACATC-3′

Electrophoretic-mobility shift assay

The binding of the nuclear extract substance to the transcription factor binding site was confirmed by an electrophoretic-mobility shift assay (EMSA). The probe, which was a 25-mer sequence (5′-GGTAATAAGTCCTGTGTTCTCC ATT-3′) containing the GRE with the complementary sequence, which is underlined, was prepared and biotinylated at the 5′ site for the probe. In addition, the probe with the mutated consensus sequence of GRE (5′-GGTAATAAGTCCCGCACTAGCCATT-3′) was constructed in the same way. A probe without biotinylation was prepared as a cold probe. A nuclear extract of rVSMCs was prepared using Panomics Nuclear Extraction Kit (Panomics, Fremont, CA, USA) after the following treatments: (1) no stimulation, (2) 10^{-7} M Aldo stimulation for 24 h, (3) 10^{-7} M Aldo stimulation for 24 h following pretreatment with 10^{-5} M Eplr for 1 h, (4) 10^{-5} M Eplr stimulation for 24 h and (5) 10^{-5} M corticosterone stimulation for 24 h. EMSA was performed with non-denatured polyacrylamide gels using a Panomics EMSA Kit (Panomics) according to the manufacturer's instructions. A supershift assay was performed with an antibody against MR (Santa Cruz Biotechnology). A LAS-3000 lumino-image analyzer (FujiFilm, Tokyo, Japan) was used to measure the fluorescence. The density of the bands was quantified using the Scion Image program. Each experiment was repeated 3–4 times.

Statistical analyses

Values are expressed as the mean ± s.e.m. Statistical comparisons were performed using analysis of variance with Scheffe's *F* procedure for *post hoc* analysis. A *P*-value <0.05 was considered as statistically significant.

RESULTS

Aldo-induced *OPN* gene transcription in rVSMCs

Aldo induced *OPN* gene transcription in a concentration-dependent manner (Figure 1a). At the concentration of 10^{-9} M, Aldo significantly increased OPN transcripts. The maximum increase was obtained at a concentration of 10^{-6} M (mean ± s.d., 2.41 ± 0.34-fold increase compared with vehicle, $P=0.016$). In addition, Aldo induced *OPN* gene transcription in a time-dependent manner (Figure 1b). OPN transcripts started to increase significantly from the 6-h time point and reached the maximum at the 24-h time point with a 2.50 ± 0.19-fold increase ($P=0.004$ compared with control). The Aldo-induced OPN transcript inductions were significantly suppressed by pretreatment with 10^{-5} M Eplr (Figure 1c).

Identification of responsible transcription factor binding sites in the *OPN* gene promoter

Luciferase activities stimulated by Aldo were measured in rVSMCs transfected with plasmids containing *OPN* promoters with various deletions. The promoter deleted to −1599 showed a statistically significant activation at the same level as the full-length promoter, but the promoters deleted to −1300 or less were not activated by Aldo (Figure 2). The GRE sequence is located between −1404 and −1386, the site to which the Aldo–MR complex might bind. The mutated promoter without the GRE sequence was not activated by Aldo. Moreover, the deletion of the consensus sequence site of GRE also resulted in a loss of the Aldo-induced activation (Figure 3). Although the consensus sequence for CREB is also located in the same area from −1460 to −1453, the CREB-deleted promoter retained the activation ability by Aldo (Figure 3).

Identification of the binding sequence in the *OPN* promoter

EMSA was conducted in order to confirm the involvement of the indicated GRE sequence in the *OPN* promoter. The nuclear extract treated with 24 h of 10^{-7} M Aldo stimulation exhibited an enhanced complex band compared with the unstimulated nuclear extract. A competition assay using cold probe showed no signal enhancement (Figure 4a). The use of a probe with a mutated GRE consensus

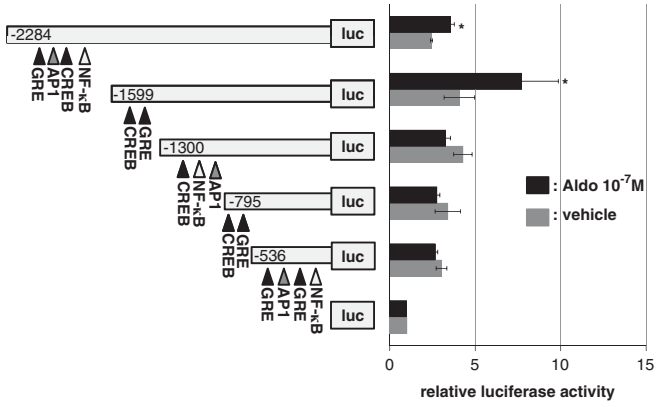

Figure 2 Identification of responsible transcription factor binding sites in the *OPN* gene promoter by luciferase assay. The promoter deleted to −1599 maintained significant activation, but the promoters deleted to −1300 or less were not activated by Aldo. The glucocorticoid response element (GRE) is located between −1404 and −1386. The promoters without the GRE sequence at −1404 lost the activation ability by Aldo. *$P<0.05$ compared with vehicle. AP-1, activator protein-1; NF-κB, nuclear factor-κB

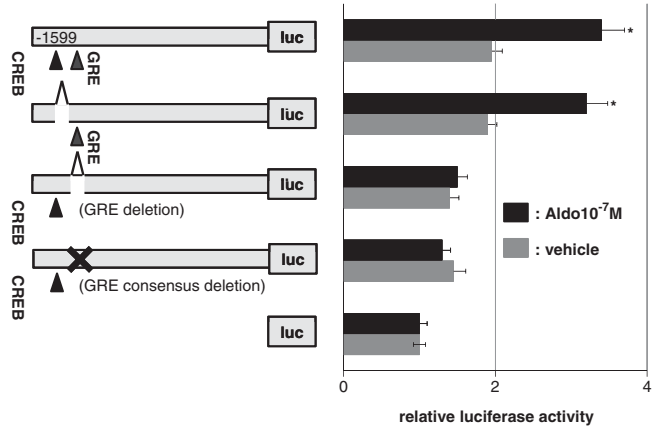

Figure 3 Identification of responsible transcription factor binding sites in the *OPN* gene promoter by luciferase assay. A mutated promoter without the GRE sequence lost the promoter activation induced by Aldo. The deletion of the GRE consensus sequence also resulted in a loss of the Aldo-induced activation. The CREB-deleted promoter retained the Aldo-induced activation. *$P<0.05$ compared with vehicle.

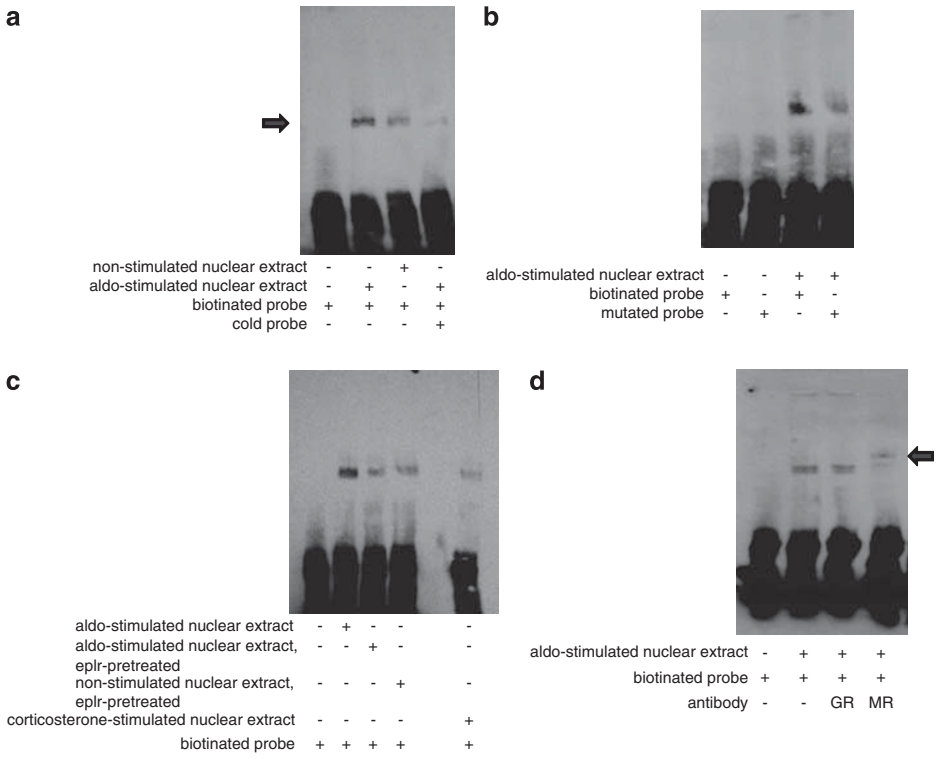

Figure 4 Electrophoretic mobility shift assay (EMSA) with nuclear extracts stimulated by Aldo. (**a**) Competition assay with cold probe. The arrow shows the location of the band of the MR-probe complex. (**b**) When using the mutated-GRE probe, the signal of the probe and nuclear protein complex was weak. (**c**) EMSA with nuclear extracts pretreated by eplerenone (Eplr) or stimulated by corticosterone. Pretreatment by Eplr made the signal weak. Stimulation by corticosterone did not enhance the signal of the complex. (**d**) Anti-MR antibody super-shifted the band (arrow). Anti-GR antibody did not shift the band. The experiments were repeated four times.

sequence (wild type: TGTGTTCT) or pretreatment with Eplr diminished the densities of the binding complex (Figures 4b and c). Stimulation with 10^{-7} M corticosterone did not increase the binding complex densities (Figure 4c). The anti-MR antibody shifted the complex bands, but anti-GR antibody did not (Figure 4d).

DISCUSSION

Although the increase of sodium reabsorption in the distal tubules was originally thought to be the main function of Aldo, several reports have shown that Aldo directly induced vascular inflammation or myocardial fibrosis.[4,25]

Aldo has been shown to induce vascular inflammation and fibrosis. However, the induction of OPN has been a well-known function of Aldo in various organs. In other words, the production of OPN was increased under various inflammatory conditions, such as that involved with calcified aortas,[17] coronary artery plaques[26] and obesity.[27] In addition, it was reported that in OPN-defective mice, fibrosis and arteriosclerotic changes were attenuated.[28,29] In this study, *OPN* gene transcription activity was enhanced dose-dependently and time-dependently in Aldo-stimulated rVSMCs, which is consistent with reports in other various cell species, such as renal mesangium cells,[19] renal fibroblasts[21] and vascular endothelial cells.[30] As the basal levels of Aldo in the plasma of humans are around 3×10^{-10} M, a significant increase in OPN transcription starting at a concentration of 10^{-9} M, as was observed in this *in vitro* analysis, seems to be within the clinical plasma Aldo fluctuation range.

Among the numerous transcription factors, there are many reports of the involvement of activator protein-1 and nuclear factor-κB in the enhancement of OPN transcriptional activity.[31–34] Although there are only a few reports on Aldo-induced OPN production, Irita *et al.*[21] reported the involvement of activator protein-1 and nuclear factor-κB in the Aldo stimulation of rat renal fibroblasts. Contrary to these past investigations, our study revealed that the formation of an Aldo–MR complex and its binding to GRE were pivotal for the upregulation of *OPN* gene transcription. Only one previous report on renal mesangium cells referred to GRE in the regulation of *OPN* gene transcription.[19] However, the GRE sequence that was used in their report was around −2000 bp from the transcription-initiating site and was an incomplete consensus sequence. GRE at −1404, the involvement of which was shown in this study, contained the complete consensus sequence that was shown to be essential to retain the OPN transcriptional upregulation by Aldo. Binding with endogenous DNA with chromatin immunoprecipitation has not yet been confirmed and hence may be examined in the future.

Glucocorticoids (cortisol in human and corticosterone in rodent) bind not only GR, but also MR, with almost the same affinity as Aldo. Considering that glucocorticoids have a 10^2–10^3-fold blood concentration compared with Aldo, glucocorticoids have to be converted to the deactivated form (cortisone in human, 11-dehydrocorticosterone in rodent) by 11β-hydroxysteroid dehydrogenase type 2 in order to retain the specific action of Aldo by the MR in rVSMCs. Although the expression of 11β-hydroxysteroid dehydrogenase type 2 is limited in the cardiovascular system,[10] Farman[35] reported that the Aldo–MR complex is more stable than the cortisol–MR complex, and the activation of gene transcription by Aldo–MR is about 100-fold higher than that activated by cortisol–MR. Furthermore, Kitagawa[36]

suggested that the altered conformation of the A/B region of MR that is induced by Aldo, but not by hydrocortisone, might determine the accessibility of the AF-1a domain of MR to RNA helicase A/CBP complexes. This report suggests that the difference in the ligands that bind to MR might be determined by the species of co-activators recruited to the MR–DNA complex.

In this study, the enhancement of the MR–DNA complex formation did not occur in EMSA, even with stimulation by corticosterone at the relatively high concentration of 10^{-5} M, however, this dose of corticosterone induced OPN transcription (data not shown). In addition, only the anti-MR antibody induced a supershift, whereas the anti-GR antibody did not. Although the precise mechanisms underlying how Aldo regulates *OPN* gene transcription are still unknown, we have clarified in this study that GRE −1401 in the OPN promoter is responsible for regulating OPN transcription through MR when stimulated by Aldo. Future studies may be needed in order to understand the mechanisms underlying how the Aldo–MR complex specifically regulates *OPN* gene expression at a finer molecular level such as that involving co-activators.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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