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Aldosterone does not Modify Gene Expression in Human Endothelial Cells

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Key words
- mineralocorticoid
- endothelial dysfunction
- genomic

Abstract

The toxic effects of aldosterone on the vasculature, and in particular on the endothelial layer, have been proposed as having an important role in the cardiovascular pathology observed in mineralocorticoid-excess states. In order to characterize the genomic molecular mechanisms driving the aldosterone-induced endothelial dysfunction, we performed an expression microarray on transcripts obtained from both human umbilical vein endothelial cells and human coronary artery endothelial cells stimulated with 10^{-7} M aldosterone for 18 h. The results were then subjected to qRT-PCR confirmation, also including a group of genes known to be involved in the control of the endothelial function or previously described as regulated by aldosterone. The state of activation of the mineralocorticoid receptor was investigated by means of a luciferase-reporter assay using a plasmid encoding a mineralocorticoid and glucocorticoid-sensitive promoter. Aldosterone did not determine any significant change in gene expression in either cell type both in the microarray and in the qRT-PCR analysis. The luciferase-reporter assay showed no activation of the mineralocorticoid receptor following aldosterone stimulation. The status of nonfunctionality of the mineralocorticoid receptor expressed in cultured human umbilical and coronary artery endothelial cells does not allow aldosterone to modify gene expression and provides evidence against either a beneficial or a harmful genomic effect of aldosterone on healthy endothelial cells.

received 15.05.2011
accepted 26.09.2011

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DOI http://dx.doi.org/10.1055/s-0031-1291272
Horm Metab Res 2011; 43: 1–5

ISSN 0018-5043

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Introduction

The position of the endothelial layer at the interface between the blood stream and the vascular wall makes it a crucial organ for maintaining vascular homeostasis in the face of continuous exogenous and endogenous stimuli. Both functional in vitro and in vivo evaluation of endothelial function have been widely-used tools to study the supposed pro-atherogenic properties of various compounds [1].

Aldosterone has been proposed as having an important role in cardiovascular pathology; it has multiple toxic effects on the vasculature and different groups have described in depth the morphological and biochemical modifications that affect the vessels in mineralocorticoid-excess states by using both animal models, primary cell cultures and in vivo evaluations of endothelial function [2]. The results of 2 clinical trials have further strengthened these data: in the Randomized Aldactone Evaluation Study [3], in patients affected by chronic heart failure, and in the Eplerenone Post-Acute Myocardial Infarction Heart Failure Efficacy and Survival Study [4], in patients with acute heart failure complicating a previous myocardial infarction, low-dose mineralocorticoid receptor (MR) antagonists given in conjunction with current best practise therapy produced a significant and relevant decrease in cardiovascular morbidity and mortality with little additional effect on blood pressure control.

In order to shed light on the molecular mechanisms driving the aldosterone-induced endothelial dysfunction and to characterize the underlying late modifications in gene expression, we performed an expression microarray analysis on transcripts obtained from both human umbilical vein (HUVEC) and coronary artery endothelial (HCAEC) cells stimulated with aldosterone for 18 h. To our surprise, we found that aldosterone did not determine any significant change in gene expression.

Materials and Methods

Cell culture

Human coronary artery endothelial cells (HCAEC), human umbilical vein endothelial cells (HUVEC), and endothelial basal and growth medium (EBM-2 and EGM-2, respectively) were purchased from Lonza. HCAEC and HUVEC were obtained at the first passage and were used between the third and sixth passages for all experiments described. In particular, the microarray analysis was performed in duplicate using transcripts obtained from cells at different passages (third and fourth passages). Cells were plated in EGM-2 containing 2% fetal bovine serum (FBS) and grown to confluence; then, medium was changed to 2% FBS-containing EGM-2 plus aldosterone 100 nM or vehicle for 18 h before RNA extraction. We did not use charcoal-stripped serum as we reasoned that those steroidal compounds contained in the 2% serum added to the basal medium would have been heavily diluted in the remaining 98% of medium, with ensuing negligible concentrations in the regular culture. Stimulation with 100 nM aldosterone should induce a preferential competitive binding of aldosterone to the MR in comparison to the other steroid hormones that display affinity for the MR itself. Aldosterone (purchased from Sigma) was added to culture medium from 1000-fold concentrated stock solutions in DMSO to give final DMSO concentrations of 0.1%. DMSO 0.1% as vehicle was added in the control.

Microarray

RNA samples used for microarray experiments were assayed for adequate purity and quality with an Agilent Bioanalyzer according to the manufacturer's instructions. The gene expression profiling platform was oligonucleotide Array Affymetrix GeneChip Human Genome U133 Plus 2.0 Array, which allows the study of >47 000 transcripts. cRNA synthesis and labeling, hybridization, and scanning were performed following the manufacturer's protocols. For each experimental condition, 2 biological replicates were included.

CEL files were analyzed using dChip 2006 Software. Briefly, the data underwent invariant set normalization in order to adjust for differences in overall array brightness. Second, expression levels of probe sets were computed using the model-based expression indexes (MBEI). We retained for further analysis only those probe sets receiving a presence (P) call across all arrays. Filtered probe sets were then ranked based on the ratio between their average experimental and baseline expression values (fold change). Transcripts with a fold change >2.0 (upregulated) or <−2.0 (downregulated) were considered as differentially expressed. Finally, hierarchical cluster analysis was performed on the full list of differentially expressed genes using correlative algorithms. For clustering, redundant probe sets were filtered.

RNA extraction, reverse transcription and polymerase chain reaction

Total RNA was extracted from cells using affinity columns (Qiagen) according to the manufacturer's protocols. RNA purity and quality was routinely checked by spectrophotometry and gel electrophoresis. Reverse transcription was performed in the presence of 100 ng/µl of total RNA, 10 ng/µl of random primers, 500 nM dNTPs, 0.1 M DTT, and 10 U/µl of SuperScript II Reverse Transcriptase (Invitrogen) in First-Strand Buffer.

The primers used to amplify the MR cDNA were: forward 5′-CAGAAATGTCTTCAAGCTGG-3′; reverse 5′-TGAGCTGTCAT AGCCTGC-3′. The amplicon length was 291 bp. The amplification conditions were: 95 °C for 1 min, 60 °C for 30 s, and 72 °C for 45 s. The cycle number was 40.

Quantitative real time-PCR (qRT-PCR) was performed in a 2-step fashion. After reverse transcription, quantitative PCR was performed using TaqMan chemistry (Applied Biosystems) on an Applied Biosystems ABI 7500 instrument following standard protocols. Gene expression levels were analyzed using the $2^{-\Delta\Delta Ct}$ relative quantification system. Predesigned Primer-TaqMan probe assays were purchased from Applied Biosystems.

Luciferase-report assay

HCAEC were transiently co-transfected (Lipofectamine 2000, Invitrogen) with 5 µg of an expression vector driving firefly luciferase under the C3H-MMTV LTR promoter at 30:1 molar ratio with the pRL vector coding for the renilla luciferase, used as an internal control of luciferase assay, for 6 h; cells were allowed to recover for 18 h and then stimulated with vehicle (DMSO 0.1%), aldosterone (1, 10, 100 nM), and cortisol (10 and 100 nM) for another 24 h. Luciferase activities were analyzed by Dual-Luciferase Report Assay System (Promega), according to vendor's instructions, using a TD20/20 double injector luminometer (Turner Designs, Forlì, IT). The results are expressed as fold activation, calculated by normalizing the ratio of the firefly/renilla luminescences.

Statistical analysis

Data from qRT-PCR experiments are expressed as mean ± SD of at least 3 independent experiments (unless otherwise stated) and differences between 2 independent variables were evaluated using a Student's *t*-test. ANOVA between groups was performed by ANOVA and the Bonferroni test was used to correct for multiple comparisons. A probability of less than 0.05 was considered statistically significant.

Results

Aldosterone does not modify global gene expression in HUVEC and HCAEC

Aldosterone has been attributed with both genomic and non-genomic effects on the vascular endothelium [5]. Aiming specifically at the late genomic effects, we performed a microarray analysis on transcripts obtained from both HUVEC and HCAEC treated with aldosterone (10^{-7} M) or vehicle for 18 h. The analysis was performed in duplicate on transcripts obtained from cells cultured at different passages.

The endothelial phenotype of the cells used for the microarray analysis was checked both by morphologic and gene expression criteria: 1) the cells displayed the typical cobblestone morphology; 2) their transcripts strongly bound probe sets coding for standard endothelial markers such as the von Willebrand factor, the platelet/endothelial cell adhesion molecule (CD31 antigen) and the endothelial protein C receptor. This preliminary check also showed the presence of the MR transcript in both HUVEC and HCAEC. The MR expression was further confirmed by performing a RT-PCR with MR-specific primers on RNA extracted from HUVEC and HCAEC belonging to the same batch (**Fig. 1**). By taking into account only those probe sets receiving a presence call across all arrays, we could not find any significant modification in gene expression induced by treatment with 10^{-7} aldosterone M both in HUVEC and in HCAEC.

Aldosterone does not modify expression of cardiovascular target genes in HCAEC

Our findings were double-checked by performing a qRT-PCR analysis on transcripts obtained from HCAECs treated under the same conditions. A group of target genes was selected on the basis either of their acknowledged role in the pathophysiology of the endothelial function or of their previously described regulation by aldosterone and the change in their expression levels was determined. Again, aldosterone did not induce any significant modification in the relative quantity of these transcripts (**Table 1**).

Further, in order to exclude the possibility that a putative genomic effect of aldosterone might have been missed by choosing the wrong time point, a time-course analysis was performed. HCAEC were stimulated for 12 and 24 h with 10^{-7} M aldosterone and the expression of some of those target genes was verified by qRT-PCR. Again, aldosterone did not induce any significant modification in the relative quantity of these transcripts (**Table 2**).

Endothelial MR is not functional

To ascertain the functional status of the MR expressed in HCAEC, we transfected these cells with a reporter plasmid driving firefly luciferase expression under the control of a MR and glucocorticoid receptor (GR)-sensitive promoter [6–8]. A second plasmid coding for renilla luciferase was co-transfected as an internal control. Stimulation with aldosterone (10^{-9} to 10^{-7} M) for 24 h did not induce any modification in the firefly/renilla luciferase activity ratio, while hydrocortisone stimulation led to a dose-dependent increase (**Fig. 2**). These data argue against a functional role of the endogenously-expressed endothelial MR and could explain the absence of effect of aldosterone stimulation we observed.

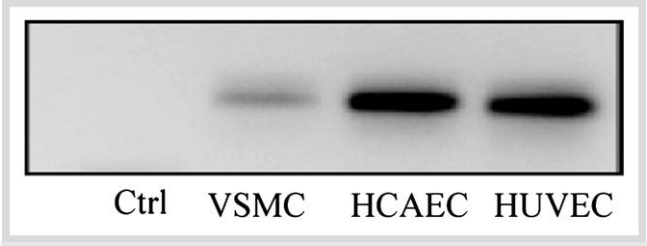

Fig. 1 MR expression in HCAEC and HUVEC. RNA was extracted from HCAEC and HUVEC and reverse transcribed to cDNA. PCR was performed (40 cycles) using MR-specific primers. Vascular smooth muscle cells (VSMC) were used as positive control and NO DNA as negative control.

Discussion

It has long been known that combining the administration of exogenous mineralocorticoids and high salt induces the development of severe hypertension and widespread lesions in the brain [9], heart [10], and kidney [11], but only in the last 2 decades the role of aldosterone as one of the major culprits in cardiovascular pathology has been recognized. A wealth of data derived from in vivo studies showed that whenever a high salt intake is combined with RAAS hyperactivity (both genetically and exogenously determined) or with direct mineralocorticoid infusion, rapid and severe target organ damage develops, which can be prevented by administering aldosterone blockers or by adrenalectomy [12–14].

Time-course experiments demonstrated that the first noxious event is inflammatory damage of blood vessels and that target organ damage was a secondary event occurring in response to ischemic/necrotic insult resulting from the vascular inflammation [15]. In a different setting, this hypothesis found confirmation in the findings of the detrimental effects of high aldosterone pathological states (heart failure and primary aldosteronism) on the endothelial function [16, 17]. Summarizing this evidence, the expression "aldosterone-induced vasculopathy" was coined to refer to the direct pathologic consequences of aldosterone action on the arterial vessel wall [18].

Hence, aiming specifically at the late genomic effects, we decided to perform a microarray analysis on transcripts obtained from HUVEC and HCAEC stimulated with aldosterone or vehicle for 18 h. In the analysis 2 main reasons led us to consider only those probe sets receiving a presence call across all arrays: 1) we considered nonsense to calculate a fold-change ratio by comparing the brightness values of probe sets which had a present call

Table 1 Aldosterone lacks effects on gene expression in human endothelial cells

Transcript	HUVEC Microarray fold change	HCAEC Microarray fold change	HCAEC qRT-PCR fold change	HCAEC qRT-PCR p-value
Angiotensin I converting enzyme (ACE)	0.9	1.1	1.1	>0.05
Angiotensin II receptor, type 1	undetected	undetected	undetermined	
Catalase	1	1	1.2	>0.05
E-selectin	1.2	1.1	0.8	>0.05
Glucose 6-phosphate dehydrogenase	1	1	1	>0.05
Heme oxygenase 1	1.05	0.95	1.1	>0.05
Intercellular adhesion molecule 1	0.9	0.9	0.7	>0.05
Nitric oxide synthase 3	1	0.9	1	>0.05
Osteopontin	undetected	undetected	undetermined	
Superoxide dismutase 1	1	1.05	1.3	>0.05
Superoxide dismutase 2	0.9	1.1	1.3	>0.05
Vascular cell adhesion molecule 1	1.1	1	0.9	>0.05

Gene expression in HUVECs and HCAECs treated with 10^{-7} M aldosterone or vehicle for 18 h was analyzed by microarray analysis and qRT-PCR

The microarray analysis was performed in duplicate; for those transcripts which are represented by a single probe set in the chip, the displayed fold change value represents the ratio between the mean value of the 2 vehicle and the 2 aldosterone-treated samples; for those transcripts, which are represented by more than 1 probe set, the displayed fold change value represents the average of the ratios between the mean value of the 2 vehicle and the 2 aldosterone-treated samples for each probe set. Only those probe sets receiving a presence call across all arrays were used in the analysis

qRT-PCR data are representative of 3 independent experiments

under 1 treatment and an absent call under the other, as the brightness coming from a probe set with an absent call has to be considered unspecific; 2) a preliminary qRT-PCR validation analysis on some of the differentially-regulated probe sets receiving a 100% presence call under one treatment and a 100% absent call under the other denied a regulation by aldosterone.

To our surprise, we found that aldosterone did not induce any significant modification in the transcription rate of any gene both in HUVEC and in HCAEC. These negative results were confirmed by qRT-PCR in HCAEC: a group of target genes which are known to have a significant role in the cardiovascular pathology was chosen and their expression levels checked at different time-points of aldosterone stimulation (12–24 h). Still, no modification could be found. Then we turned our attention to the functional status of the endothelial MR by performing a luciferase-reporter assay, which showed no MR activation following aldosterone stimulation. In setting up these experiments, we were fully aware that 10^{-7} M aldosterone is a supraphysiologic concentration; still, we decided to retain it considering that most publications investigating the in vitro transcriptional effects of aldosterone on endothelial cells showed 10^{-7} M aldosterone exerting the greatest effects [19–21]. Similarly, the choice of the 18-h time-point was made bringing in mind that the other authors had used incubation times ranging from 12 to 24 h [8, 19–21]. Further, we confirmed the expression of the MR at the mRNA level in the batches of HUVEC and HCAEC that we used for the experiments, in accordance with Caprio et al. who had already given clear and exhaustive demonstration of its presence in HUVEC and HCAEC both at the mRNA and at the protein level [8].

Nonetheless, we acknowledge as a potential limitation of our study the use of unstripped serum, which could have made MR less available for binding by aldosterone, even at high concentrations, due to the other steroidal compounds present in the medium.

We can only speculate on the reasons behind the discrepancies between our data and the literature. In a recent in vivo study performed on young volunteers, Nietlispach et al. [22] found that a state of artificially induced mineralocorticoid excess obtained by 14 days of oral intake of fludrocortisone induced an enhancement of the endothelium-dependent vasodilation of the forearm vasculature through an increase in the basal NO availability. Previously, other authors had reported the same findings in the setting of acute aldosterone infusions [23, 24]. As a possible interpretation of these results, the noxious vascular effects of mineralocorticoids might take place only in the presence of preexisting classical or nonclassical cardiovascular risk factors, which would play a permissive role for subsequent MR activation and vascular damage. Partial experimental confirmation of this theory has come from a paper describing the inability of aldosterone to induce oxidative stress and vascular inflammation in mice with reduced monocyte/macrophage function [25]; further, Nguyen Dinh Cat et al. [26] recently reported that selective MR overexpression in the endothelium of healthy mice was not able to induce any sign of endothelial dysfunction, vascular inflammation or alteration in the morphology of the vessel wall. The putative mechanism triggering MR activation under preexisting noxious circumstances remains to be elucidated, but it is tempting to link this theory to the hypothesis that under normal conditions, glucocorticoids occupy the MR in a tonic inhibitory mode (explaining the results of the luciferase assay), but act as agonists in situations of tissue damage, reactive oxygen species generation, or inappropriate salt status [27]. Alternatively, we cannot exclude the possibility that aldosterone could induce post-transcriptional or post-translational modifications of its targets or act through indirect mechanisms (i.e., by inducing hypokalemia) which, given our experimental setting, would have been missed. Overall, the in vitro investigation of aldosterone effects on endothelial function may be lacking in at least one of several possible mechanisms, such as chronic elevated aldosterone concentrations, high blood pressure, high sodium, low potassium, other concomitant damaging factors, such as the oxidative stress induced by monocytes, that are present in animal models and patients with primary and secondary aldosteronism, that are indispensable and permissive for aldosterone to play its role causing endothelial dysfunction.

These hypothesis and our data contrast with the results of previous studies conducted on cultured endothelial cells, which have shown the ability of aldosterone to act through genomic pathways to induce endothelial damage either by increasing genera-

Table 2 Time-course analysis of gene expression in aldosterone-treated HCAEC

Transcript	HCAEC qRT-PCR fold change		
	12 h	18 h	24 h
Angiotensin I converting enzyme (ACE)	1	1.1	1.1
Glucose 6-phosphate dehydrogenase	0.9	1	0.9
Intercellular adhesion molecule 1	0.8	0.7	0.9
Nitric oxide synthase 3	1	1	1
Vascular cell adhesion molecule 1	0.9	0.9	0.8

Gene expression in HCAECs treated with 10^{-7} M aldosterone or vehicle for 12, 18, or 24 h was analyzed by qRT-PCR. p-Value is >0.05 in all cases

Data are representative of 3 independent experiments

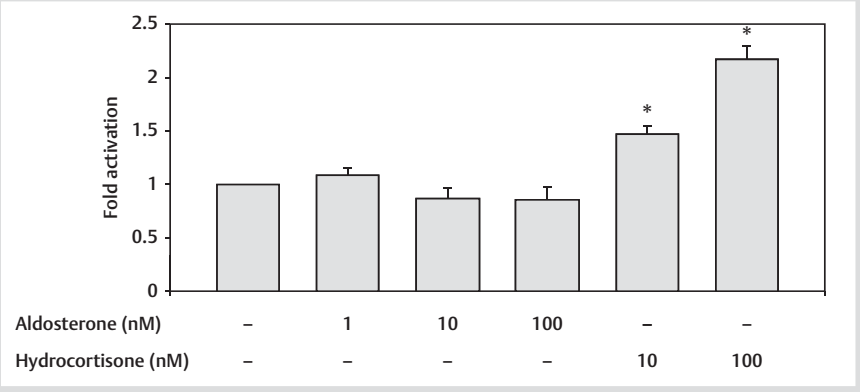

Fig. 2 Functional status of the MR in HCAEC. HCAEC were co-transfected with an MR and GR-driven luciferase expression vector at a 30:1 molar ratio with the pRL vector coding for the renilla luciferase as an internal control and stimulated with aldosterone or hydrocortisone for 24 h as indicated. Results are expressed as fold activation relative to vehicle-treated cells, calculated by normalizing the ratio of the firefly/renilla luminescences. Data are representative of 3 independent experiments. *$p<0.05$ vs. vehicle-treated cells.

tion of reactive oxygen species [19,28] or by inducing the expression of markers of inflammation [8,20] or of proteins involved in the vascular damage [21]. At present, the reason for such discrepancies is not clear.

In summary, our data indicate that aldosterone does not induce a late modification of gene expression in cultured endothelial cells and do not support either a beneficial or a harmful genomic effect of aldosterone on endothelial function.

Acknowledgements

We would like to thank Dr. Daniele Tomassoni and Dr. Seyed Khosrow Tayebati, University of Camerino, Italy, for constructive comments and discussion of the results and reviewing the manuscript.

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