

# Human Endothelium: Target for Aldosterone

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**Abstract**—Aldosterone has long been known to control water and electrolyte balance by acting on mineralocorticoid receptors in kidney. However, recent studies demonstrated the presence of these receptors in nonclassical locations, including the cardiovascular system. We tested the hypothesis whether endothelial cells respond to aldosterone with changes in cell volume, a measure for ion-mediated water movement across the cell membrane. By means of atomic force microscopy in fluid, we measured volume of adherent human umbilical venous endothelial cells exposed for 72 hours to 10 nmol/L aldosterone. Over this period of time, cells swell by  $\approx 18\%$ . Aldosterone-induced swelling is prevented by 100 nmol/L of the mineralocorticoid receptor antagonist spironolactone, added to the primary endothelial cell culture. Aldosterone-treated cells dramatically shrink when 1  $\mu\text{mol/L}$  of the diuretic amiloride is applied. Cells deprived of aldosterone do not respond to amiloride. Our conclusions are: (1) aldosterone leads to sustained cell swelling inhibited by administration of spironolactone or the sodium channel blocker amiloride; (2) cells respond to amiloride after aldosterone exposure; (3) renal diuretics act on endothelial cells; and (4) both amiloride and spironolactone could be useful for medical applications to prevent aldosterone-mediated endothelial dysfunction. (*Hypertension*. 2004; 43:952-956.)

**Key Words:** endothelium ■ mineralocorticoids

The kidney is known to be the major target for aldosterone, a mineralocorticoid hormone synthesized in the adrenal cortex that acts on electrolyte transport in the distal nephron.<sup>1</sup> However, there is strong evidence that this hormone is also synthesized in heart<sup>2</sup> and blood vessels.<sup>3</sup> At these locations, it is regulated by similar mechanisms comparable to the renin-angiotensin aldosterone system.<sup>4,5</sup> Because of the fact that aldosterone acts on cardiomyocytes, cardiac fibroblasts, and endothelial cells, this hormone plays a major role in the development of heart failure, myocardial fibrosis, and endothelial dysfunction.<sup>6</sup> Moreover, there is much interest in the possibility of the use of aldosterone receptor blockade in patients to diminish pathological effects that can be produced by this hormone.<sup>7</sup>

A study applying atomic force microscopy (AFM) on living aortic endothelial cells showed transient cell swelling that occurred over minutes and that was prevented by a high dose of amiloride known to inhibit plasma membrane  $\text{Na}^+/\text{H}^+$  exchange.<sup>8</sup> Although the underlying mechanism and its physiological relevance were still unclear, attention was placed on data suggesting that endothelial cells not only synthesize aldosterone<sup>3</sup> but also express mineralocorticoid receptors<sup>9</sup> and the epithelial sodium channel.<sup>10</sup> In a recent article, we applied cariporide, a specific  $\text{Na}^+/\text{H}^+$  exchange inhibitor, to human umbilical venous endothelial cells (HUVECs).<sup>11</sup> To our surprise, we found that the specific  $\text{Na}^+/\text{H}^+$  exchange inhibitor did not prevent aldosterone-induced cell swelling,

whereas, in contrast, a low dose of amiloride known to block plasma membrane sodium channels was most effective. Taken together, these observations indicated that aldosterone triggers the “classic” signal cascade in endothelial cells, similar to that in the kidney. To test for the classic (ie, long-term) response in a reliable endothelial cell preparation, we performed experiments in primary HUVEC cultures exposed to aldosterone over a time period of 72 hours. Primary HUVEC cultures express the mineralocorticoid receptors<sup>9,11</sup> and closely reflect endothelium in vivo.<sup>12,13</sup>

We assumed that, similar as in target cells of the kidney, aldosterone should alter cellular ion and water transport and thus change cell volume in adherent HUVECs measured by atomic force microscopy. We found that HUVECs share some major characteristics with principal cells of the renal collecting duct, including their response to diuretics.

## Methods

### Endothelial Cell Culture

HUVECs were grown in culture as described.<sup>12,13</sup> In short, cells (passage p0) were cultivated in  $T_{25}$  culture flasks coated with 0.5% gelatin (Sigma-Aldrich Chemie GmbH, Steinheim, Germany). After reaching confluence, cells were split using trypsin and then cultured (passage p1) on thin (diameter=15 mm) glass coverslips coated with 0.5% gelatin and cross-linked with 2% glutaraldehyde. Glass coverslips were placed in petri dishes filled with culture medium. HUVECs formed confluent monolayers within 72 hours (at 37°C, 5%  $\text{CO}_2$ ). Chemicals were added to the medium as appropriate. All

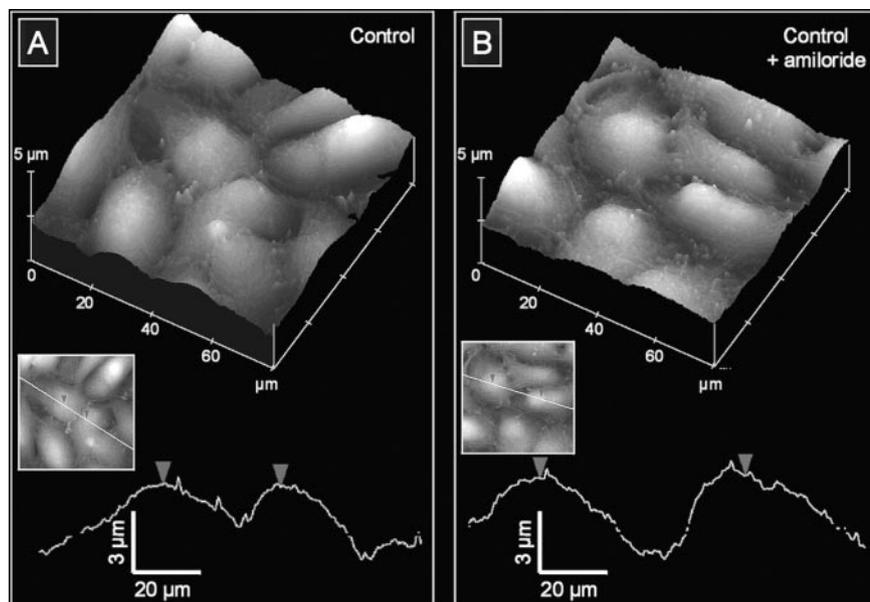
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**Figure 1.** AFM images and cell profiles of HUVECs. Lines in the insets correspond to the respective profiles. Triangles indicate individual locations for orientation. A, HUVEC cultured in medium for 72 hours in absence of aldosterone. B, HUVEC cultured in medium for 72 hours in absence of aldosterone with addition of amiloride 60 minutes before fixation.

procedures (as addition of diuretics and fixation by glutaraldehyde) were performed in the incubator. Aldosterone (d-aldosterone; Sigma-Aldrich Chemie GmbH) was dissolved in ethanol (stock solution=1 mmol/L, stored at 4°C for 2 weeks). Final concentration in the experiments was 10 nmol/L. Spironolactone (ICN Biochemicals GmbH, Eschwege, Germany) was dissolved in ethanol (stock solution=1 mmol/L) and applied with a final concentration of 100 nmol/L. Furthermore, we used the epithelial Na<sup>+</sup> channel blocker amiloride (Sigma-Aldrich), dissolved in water, at a final concentration of 1 μmol/L. In corresponding control experiments, we added only the solvents (ethanol 0.1% or water) to the media. After appropriate time, HUVECs were fixed by glutaraldehyde (final concentration=0.5%), gently added to the medium.

### AFM Cell Volume Measurements

The method of endothelial cell volume determination by AFM has been described in detail.<sup>14</sup> AFM was performed in fluid using a Nanoscope III Multimode-AFM (Digital Instruments, Santa Barbara, Calif) with a J-type scanner (maximal scan area:100×100 μm). V-shape oxide-sharpened cantilevers with spring constants of 0.06 N/m (Digital Instruments) were used for scanning in fluid. Surface profiles (512×512 pixels) were obtained with scan sizes of 10 000 μm<sup>2</sup> at a scan rate of 6 Hz. Further settings were height mode, gains between 6 and 10, interaction force between AFM tip, and sample surface <5 nN. Five to 10 images were obtained from individual samples and analyzed using the Nanoscope III software (Digital Instruments). Each image was plane-fitted (order 1) and the volume of the total image (≈7 to 12 cells per image) was analyzed using the “bearing” software feature. A mean single cell volume was obtained by dividing the total monolayer volume by the number of cells.

### Results

HUVECs were grown to confluence over a time period of 72 hours. Cells, most cautiously fixed under physiological conditions (ie, in culture medium with fetal calf serum, inside the incubator, at 37°C and 5% CO<sub>2</sub>), were scanned in fluid and cell volumes were calculated.

Under aldosterone-free (control) conditions, single-cell volume averages 1652±63 fL. Addition of 1 μmol/L amiloride to the primary HUVEC culture, 60 minutes before fixation, does not significantly affect cell shape or cell volume (Figure 1). Profiles taken from some of the cells confirm this observation.

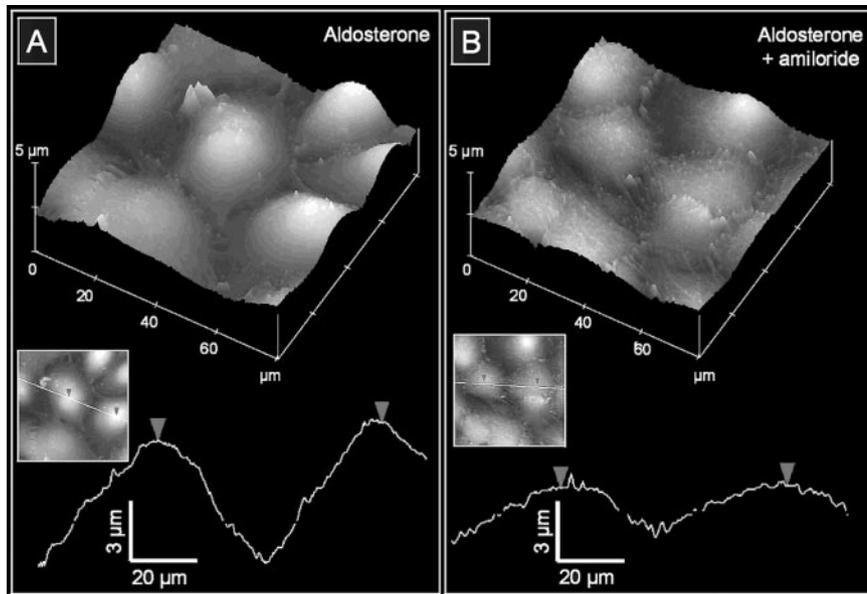
However, HUVEC undergo changes in shape and volume when exposed to aldosterone (Figure 2). Single cell volume is found significantly increased to 1996±161 fL. The surface images and the corresponding profiles reflect the increase in volume. Under these conditions, amiloride is effective. The diuretic dramatically decreases cell volume to 1384±25 fL. Cell profiles underline this observation. As a consequence of shrinkage, cell surface becomes ruffled.

To test whether swelling, induced by aldosterone, was mediated through the genomic pathway, we coincubated HUVECs with 10 nmol/L aldosterone and its receptor antagonist, spironolactone (100 nmol/L). Spironolactone prevents cell swelling elicited by aldosterone (Figure 3). Under these conditions, single cell volume averages 1672±69 fL. When mineralocorticoid receptors are functionally knocked out by spironolactone over 72 hours, not only is aldosterone ineffective, but also the sodium channel blocker amiloride. The response of HUVEC volume to this diuretic is weak. HUVEC volume averages 1524±62 fL. This value is not significantly different from the corresponding mean value obtained in absence of amiloride. Cell profiles obtained in presence and absence of amiloride appear similar.

Figure 3 summarizes the results. Aldosterone increases HUVEC volume. Concomitant exposure of HUVEC to spironolactone prevents aldosterone-induced cell swelling. Acute administration of amiloride (1 μmol/L for 60 minutes) shrinks aldosterone-exposed cells but is without effect in aldosterone nonexposed cells. In other words, HUVECs gain sensitivity to amiloride by aldosterone treatment.

### Discussion

In sodium reabsorbing epithelia, it has been well established that aldosterone activates plasma membrane sodium channels.<sup>15</sup> Aldosterone elicits cellular responses in human endothelial cells similar as compared with cells of the renal collecting duct. In kidney, the major target is the principal cell of the distal nephron where systemic sodium deprivation, and hence increase of plasma aldosterone concentration,



**Figure 2.** AFM images and cell profiles of HUVECs. Lines in the insets correspond to the respective profiles. Triangles indicate individual locations for orientation. A, HUVEC cultured in medium for 72 hours in presence of aldosterone. B, HUVEC cultured in medium for 72 hours in presence of aldosterone with addition of amiloride 60 minutes before fixation.

stimulates amiloride-sensitive electrical currents as measured by patch-clamp techniques.<sup>16</sup> These currents are caused by active sodium channels in apical plasma membrane that are activated by aldosterone either through yet unknown non-genomic mechanisms<sup>17</sup> or through classic genomic mechanisms using intracellular mineralocorticoid receptors.<sup>18</sup>

The present data obtained in HUVECs indicate a genomic effect of aldosterone, derived from the fact that the mineralocorticoid receptor antagonist spironolactone blocked the response to the hormone. HUVECs swell in response to the hormone, a phenomenon inhibited by the sodium channel blocker amiloride. The HUVEC preparation is a primary

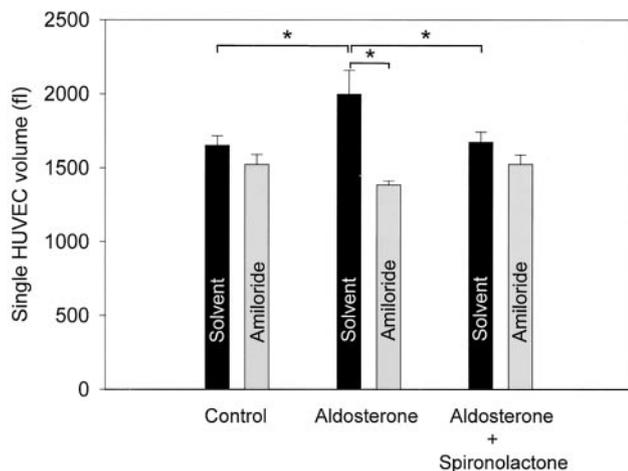
culture that retains its specific characteristics in terms of endothelial structure and function.<sup>12,13</sup>

How is aldosterone-induced cell swelling and amiloride-induced cell shrinkage explained? HUVECs are known to express mineralocorticoid receptors<sup>9</sup> and plasma membrane sodium channels.<sup>10</sup> Generally, activation causes receptors to translocate into the nucleus where gene transcription is initiated. After some time delay that can be highly variable in length and depend on cell type and experimental conditions, functional sodium channels appear in the apical cell membrane.

This sequence of events is likely to occur not only in renal target cells but also in cells of the cardiovascular system including endothelial cells.<sup>6</sup> In any case, induction of sodium channels causes cells to electrically depolarize because of increased sodium influx. Although sodium is pumped out of the cell by the Na<sup>+</sup>/K<sup>+</sup>ATPase, located in the basolateral membrane, Cl<sup>-</sup> accumulates in the cell because of electrochemical driving forces set by the altered cell membrane potential. This causes intracellular retention of water and cell swelling.

The amiloride response can be explained in a similar way. It is evident that only cells with significant sodium permeability, based on epithelial sodium channel activity, can respond to micromolar amiloride concentrations. Such increased sodium permeability exists in HUVECs after aldosterone treatment. Under these conditions, application of amiloride blocks the apical sodium channels and thus hyperpolarizes the cell membrane. Electrochemical driving forces lead to Cl<sup>-</sup> efflux. Concomitantly, water leaves the cell and shrinkage occurs. This explains the observation that HUVEC volume is insensitive to amiloride before aldosterone exposure but becomes amiloride-sensitive after aldosterone treatment.

Amiloride is a potassium-sparing diuretic that clearly acts on sodium channels of kidney tubules.<sup>16</sup> At usual daily dosages of 5 to 10 mg, amiloride reaches micromolar concentrations in renal collecting ducts, caused by renal concen-



**Figure 3.** Single HUVEC volume obtained after 72 hours of incubation. Over the whole time period, 10 nM aldosterone and 100 nM spironolactone were present. Sixty minutes before fixation, 1 μmol/L amiloride (gray bar) or solvent (black bar) were added. Twenty AFM images were analyzed per column. Mean values are given as columns ±SEM. Student Newman-Keuls method was applied for multiple testing statistical significance between experiments from different groups (control group, aldosterone group, and aldosterone+spironolactone group). The Mann-Whitney rank sum test was used for testing statistical significance within groups (amiloride versus solvent). \*Significant, P=0.05.

trating mechanisms.<sup>19</sup> Such concentrations are high enough to block the luminal sodium channels. In peripheral blood, concentrations are 10- to 100-times lower, possibly too low to affect the sodium channels of the endothelium.<sup>20</sup> Nevertheless, a single amiloride injection could transiently hyperpolarize endothelial cells of a patient with aldosteronism and thus could lead to a transient shift of potassium from blood to endothelium.

Endothelial cell swelling is a phenomenon observed in ischemia<sup>21,22</sup> and lactacidosis.<sup>23</sup> It is based on the activation of the plasma membrane  $\text{Na}^+/\text{H}^+$  exchanger inhibited by amiloride analogues as ethylisopropylamiloride. Although aldosterone can exert rapid responses through activation of  $\text{Na}^+/\text{H}^+$  exchange<sup>24–27</sup> and can swell endothelial cells,<sup>23</sup> this is not the mechanism of aldosterone-induced HUVEC swelling we describe here. Micromolar amiloride concentrations do not affect the activity of the  $\text{Na}^+/\text{H}^+$  exchanger to a significant extent, but completely block epithelial sodium channels. Furthermore, cariporide, a specific  $\text{Na}^+/\text{H}^+$  exchange blocker, turned out to be ineffective when applied instead of amiloride.<sup>11</sup> This indicates that cell swelling is associated with increased  $\text{Na}^+/\text{H}^+$  exchange activity only if cells are challenged by an anaerobic metabolism including intracellular acidosis. This is not the case after aldosterone treatment. Rather, intracellular alkalosis is expected because of the favorable inward-directed electrochemical driving forces for  $\text{HCO}_3^-$ , induced by aldosterone-mediated cell depolarization (caused by activation of sodium channels).

Hypokalemic alkalosis and hypertension characterize the syndrome of primary aldosteronism.<sup>28</sup> The prevalence of primary aldosteronism in patients with hypertension is in the range of 5% to 13%.<sup>28–30</sup> Hypokalemia and hypertension have been attributed to the aldosterone-induced altered function of the kidney. Aldosterone activates sodium and potassium channels in principal cells of distal tubule and collecting duct, leading to sodium reabsorption and potassium secretion.<sup>31</sup> In parallel, acid equivalents are secreted in the distal nephron.<sup>32</sup>

However, aldosterone acts not only on epithelial cells of kidney and colon but also at nonepithelial sites in the brain, heart, and vasculature.<sup>6</sup> We emphasize the role of endothelial cells in the physiology and pathophysiology of aldosterone action. Because endothelial cells respond to aldosterone in a similar way as renal epithelial cells, they should help to retain sodium in the organism and to release potassium. We postulate that at physiological aldosterone concentrations, endothelial cells support the kidney in rapidly adjusting extracellular volume and plasma potassium concentration. In aldosteronism, endothelial cells can release large amounts of potassium into the blood because of cell depolarization caused by the aldosterone-activated sodium channels. Because the total volume of endothelial cells in the adult human organism is in the same range as total blood volume ( $\approx 5$  L), and because total endothelial cell surface is extremely large ( $\approx 1000$  m<sup>2</sup>), there is a huge potassium buffer available that can be shifted rapidly.

Definition of the effective “physiological” concentration of aldosterone is difficult. Dose–response curves for a “chronic” aldosterone response are not feasible, possibly because of the

fact that the “systemic” aldosterone concentration (in plasma) most likely differs from “local” concentration (eg, paracrine secretion). In a previous study, we needed 100 nM of aldosterone to exert transient changes in HUVEC volume (ie, transient changes over minutes).<sup>11</sup> In the present study, we needed 10 nM of aldosterone to exert sustained changes in cell volume (ie, sustained changes over days). We conclude that the issue of the effective aldosterone concentration in endothelial cells is complex and still unresolved.

### Perspectives

This view opens new perspectives concerning treatment of diseases that involve high aldosterone concentrations. Spironolactone and analogues have recently turned out to be extremely useful in the treatment of cardiovascular dysfunctions.<sup>28,29,33</sup> This has been mainly attributed to the antagonizing action of the mineralocorticoid receptor antagonists in cardiomyocytes and cardiac fibroblasts. From the present data, we derive that a major target for aldosterone is the endothelium that could significantly contribute to cardiovascular dysfunction when aldosterone is elevated in the organism.

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