

Endothelial Cell Swelling by Aldosterone

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Received: 7 May 2003/Revised: 7 October 2003

Abstract. There is accumulating evidence that mineralocorticoids not only act on kidney but also on the cardiovascular system. We investigated the response of human umbilical venous endothelial cells (HUVECs) to aldosterone at a time scale of 20 minutes in absence and presence of the aldosterone antagonist spironolactone or other transport inhibitors. We applied atomic force microscopy (AFM), which measures cell volume and volume shifts between cytosol and cell nucleus. We observed an immediate cell volume increase (about 10%) approximately 1 min after addition of aldosterone (0.1 $\mu\text{mol/l}$), approaching a maximum (about 18%) 10 min after aldosterone treatment. Cell volume returned to normal 20 min after hormone exposure. Spironolactone (1 $\mu\text{mol/l}$) or amiloride (1 $\mu\text{mol/l}$) prevented the late aldosterone-induced volume changes but not the immediate change observed 1 min after hormone exposure. AFM revealed nuclear swelling 5 min after aldosterone addition, followed by nuclear shrinkage 15 min later. The Na^+/H^+ exchange blocker cariporide (10 $\mu\text{mol/l}$) was ineffective. We conclude: (i) Aldosterone induces immediate (1 min) swelling independently of plasma membrane Na^+ channels and intracellular mineralocorticoid receptors followed by late mineralocorticoid receptor- and Na^+ -channel-dependent swelling. (ii) Intracellular macromolecule shifts cause the changes in cell volume. (iii) Both amiloride and spironolactone may be useful for medical applications to prevent aldosterone-induced vasculopathies.

Key words: HUVECs — Atomic force microscopy — Cariporide — Mineralocorticoids — Na^+ channel — Vasculopathies

Introduction

In humans, the kidney is known to be the major target for aldosterone, a mineralocorticoid hormone synthesized in the adrenal cortex and acting upon electrolyte transport in the distal nephron (Berliner & Giebisch, 2001; Rossier, 2002). However, there is increasing evidence that this hormone is also synthesized in the heart (Silvestre et al., 1998) and blood vessels (Takeda et al., 1995) and that this is regulated by similar mechanisms as the renin-angiotensin-aldosterone-system reviewed recently (Lijnen & Petrov, 2000; Epstein, 2001). Furthermore, aldosterone is supposed to act directly at the site of synthesis, a view that is strongly supported by experimental as well as clinical data. Due to the fact that aldosterone acts on cardiomyocytes, cardiac fibroblasts and endothelial cells, this hormone seems to play a major role in the development of heart failure, myocardial fibrosis and endothelial dysfunction (Stier, Jr., Chander & Rocha, 2002). 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 (Palmieri, Biondi & Fazio, 2002). Some years ago it was shown that aldosterone can elicit a fast non-genomic response in endothelial cells. This manifests itself with an acute increase of intracellular calcium (Schneider et al., 1997a). A study applying atomic force microscopy (AFM) to living aortic endothelial cells showed transient cell swelling that occurred within minutes and that was prevented by amiloride (Schneider et al., 1997b). At the time, the physiological relevance of these data was unclear. However, it is evident that endothelial cells not only synthesize aldosterone (Takeda et al., 1995) but also express mineralocorticoid receptors (Lombes et al., 1992) and the epithelial sodium channel (Golestaneh et al., 2001), which is itself typically expressed in aldosterone-responsive cells. Experiments

in our own laboratory shed new light on the genomic signaling pathway of aldosterone. We observed with AFM at the single-molecule level in hormone-injected oocytes that aldosterone-induced receptor/mRNA translocation between cytosol and nucleoplasm involves major macromolecule movements within narrow time slots (Schafer et al., 2002). This led to the assumption that intracellular movements in macromolecules should be accompanied by volume shifts in the respective intracellular compartments. Therefore, we developed a method to measure the volume of adherent cells in culture. By using AFM, such measurements are possible, independent of the actual cell shape. In addition, we performed electronic cell sectioning that makes it possible to distinguish a nuclear volume change from a total cell volume change. For the experiments we chose primary cultures of human umbilical venous endothelial cells and we could detect transient volume changes induced by aldosterone. The mineralocorticoid receptor and Na^+ -channel blockers could not prevent initial cell swelling but were effective a few minutes later, indicating dual action of the hormone on HUVECs.

Materials and Methods

ENDOTHELIAL CELL CULTURE

Human umbilical venous endothelial cells (HUVECs) were isolated and grown in culture as described (Jaffe et al., 1973; Goerge et al., 2002). The culture medium (M199, Gibco, Karlsruhe, Germany) contained 10% heat-inactivated fetal calf serum (Roche, Mannheim, Germany), antibiotics (penicillin 100 U/ml, streptomycin 100 $\mu\text{g}/\text{ml}$), 5U/ml heparin (Biochrom KG, Berlin, Germany) and 1 ml/100 ml growth supplement derived from bovine retina as described (Golestaneh et al., 2001). Cells (passage p0) were cultivated in T₂₅ culture flasks coated with 0.5% gelatine (Sigma-Aldrich Chemie, Steinheim, Germany). After reaching confluence, cells were split using trypsin and then cultured (passage p1) on thin glass coverslips (diameter = 15 mm) coated with 0.5% gelatine and cross-linked with 2% glutaraldehyde. Glass coverslips were placed in Petri dishes filled with culture medium. After HUVECs formed confluent monolayers (within 72 h at 37°C, 5% CO₂) chemicals were added to the medium as appropriate. This procedure, including fixation by glutaraldehyde, was performed in the incubator and particular care was taken to minimize changes in ambient temperature or CO₂ and to avoid any shear stress applied to the adherent HUVEC monolayer during manipulations. Aldosterone (d-aldosterone, Sigma-Aldrich) was dissolved in ethanol (stock solution = 1 mmol/l, stored at 4°C for two weeks). Final concentration in the experiments was 100 nmol/l. Spironolactone (ICN Biochemicals GmbH, Eschwege, Germany) was dissolved in ethanol (stock solution = 1 mmol/l) and applied with a final concentration of 1 $\mu\text{mol}/\text{l}$. Spironolactone was added 10 minutes prior to aldosterone to make sure that virtually all mineralocorticoid receptors were occupied before cells were exposed to the agonist (aldosterone). Furthermore, we used the plasma membrane Na^+/H^+ exchange inhibitor cariporide (gift of Aventis Pharma Deutschland, Frankfurt, Germany), dissolved in water (stock solution = 1 mmol/l), at a final concentration of 10 $\mu\text{mol}/\text{l}$ and the epithelial Na^+ -channel blocker amiloride (Sigma-Aldrich), dissolved in water at increased temperature (about 40°C), at a final

concentration of 1 $\mu\text{mol}/\text{l}$. Both inhibitors were applied similarly to the protocol mentioned above for Spironolactone treatment. In corresponding control experiments we added only the solvents (ethanol 0.1%) to the media. After appropriate time periods HUVECs were fixed with glutaraldehyde (final concentration = 0.5%) gently added to the medium. Cells remained undisturbed for the next 45 minutes in the fixative in the incubator. Then the medium was exchanged by HEPES-buffered solution (mmol/l: 140 NaCl; 5 KCl; 1 MgCl₂; 1 CaCl₂; 10 HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid); pH = 7.4) and HUVECs were stored in HEPES buffer under sterile conditions at 4°C. Fixed HUVECs could be kept in fluid for at least a week without measurable changes in morphology or cell volume.

AFM CELL VOLUME MEASUREMENTS

Glass coverslips were attached to stainless steel disks with double-sided adhesive tape and mounted in the commercially available fluid cell of the atomic force microscope. Care was taken to keep the HUVECs in fluid at all times. AFM was performed in contact mode using a Nanoscope III Multimode-AFM (Digital Instruments, Santa Barbara, California, USA) with a J-type scanner (maximal scan area: 100 × 100 μm). V-Shaped 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^2 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, less than 5 nN. 5 to 10 images were obtained from individual samples and analyzed using the Nanoscope III software (Digital Instruments). First, the individual image was plane-fitted (order 1) and then, the volume of the total image (about 7 to 12 cells per image) analyzed using the “bearing” software feature. To detect changes in nuclear volume we electronically cut the entire image in slices and separately evaluated the top slice taken at half height of the cell monolayer (Fig. 1). We assumed that any changes in nuclear volume should be reflected by volume changes of the top, since this part of the monolayer should represent mainly nucleoplasm, whereas the base part of the monolayer should mainly represent the cytosol. A mean single-cell volume was obtained by dividing the total monolayer volume by the number of cells. The method of measuring cell volume of fixed adherent HUVECs by AFM was tested in a classical approach applying hypotonic solution over a period of 5 minutes (see Fig. 3). We measured cell volume 30 s, 1, 2 and 5 min after exposure to hypotonic solution (reduction of the osmolarity by 20% adding H₂O to the culture medium). These experiments were performed identically to the protocols described above.

RT-PCR ANALYSIS OF MINERALOCORTICOID RECEPTOR EXPRESSION

Total RNA of HUVECs was prepared using Trizol reagent (Gibco BRL) according to the manufacturer's instructions (2.5 ml of Trizol per 2.5×10^6 cells). RNA was quantified and its quality assessed by spectrophotometry. In order to remove contaminating DNA, RNA was incubated with 20 U DNase I (MBI Fermentas, St. Leon-Rot, Germany) per μg RNA for 30 min at 37°C. After DNase I digestion and phenol-chloroform-isoamylalcohol extraction, 5 μg of total RNA were used per reverse transcription reaction. Complementary DNA (cDNA) synthesis was performed with Superscript II reverse transcriptase (Gibco BRL) according to manufacturer's instructions and 500 ng oligo(dT)₁₅ (Promega, Madison, WI) per reaction (20 μl) as first-strand primer. cDNA was purified by phenol-chloroform-isoamylalcohol extraction and re-dissolved in 20 μl sterile,

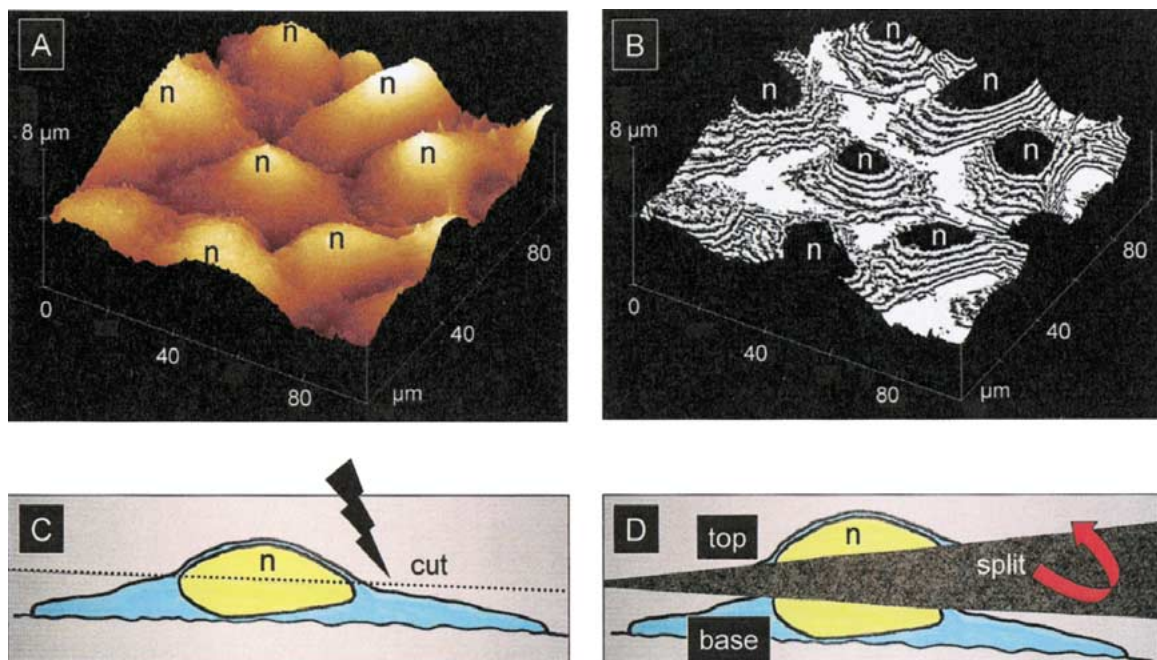


Fig. 1. Atomic force microscopy (AFM) on human umbilical venous endothelial cells (HUVECs) and method of electronic sectioning. (A) AFM image of HUVECs grown on gelatine-coated support, fixed at physiological conditions and scanned in buffered solution, n = nuclear compartment. (B) Same image as shown at left (A), with height profiles. Black tops indicate the nuclear com-

partments (n) of individual HUVECs. (C) Schematic of a single cell with section line at half height. (D) Cell is electronically split into a top part that mainly represents the nuclear compartment, and into a base part that mainly represents the cytosolic compartment. The volume of the respective parts can be separately analyzed.

distilled water. A 900-bp fragment of human mineralocorticoid receptor was amplified by polymerase chain reaction (PCR) using primers 5'-TCAAGTCCGTTAAGTAGCAT-3' and 5'-TTTCCATGACTCCACTAAAG-3'. The reaction mixture consisted of 3 μ l cDNA, 1.25 U *Taq* DNA polymerase (New England Biolabs, Frankfurt, Germany), 200 nmol/l of each primer, 200 μ mol/l dNTPs, 10 mmol/l KCl, 10 mmol/l $(\text{NH}_4)_2\text{SO}_4$, 20 mmol/l Tris-HCl pH 8.8, 2 mmol/l MgSO_4 and 0.1% Triton X-100 in a final volume of 50 μ l. PCR was performed in a thermal cycler (Mastercycler Gradient, Eppendorf, Hamburg, Germany) with an initial denaturation at 94°C for 5 min, 35 cycles of denaturation (94°C, 1 min), annealing (59°C, 1 min) and synthesis (72°C, 1 min 30 sec) and 15 min of final extension at 72°C. Reverse transcription reactions with water added instead of reverse transcriptase were used as negative control templates. PCR products were resolved on 1.5 % agarose gels in 0.5 \times TBE (44.5 mmol/l Tris, 44.5 mmol/l boric acid, 1 mmol/l EDTA), stained in 1 \times SYBR Gold (Molecular Probes, Leiden, Netherlands) in 0.5 \times TBE and photographed on a UV transilluminator.

STATISTICAL ANALYSIS

Mean data of experiments are given \pm standard error of the mean (SEM). Statistical significance was tested with unpaired Students *t*-test. Significantly different is $P < 0.05$ or less.

Results

In a first step we tested whether HUVECs express the mineralocorticoid receptor. As indicated in Fig. 2,

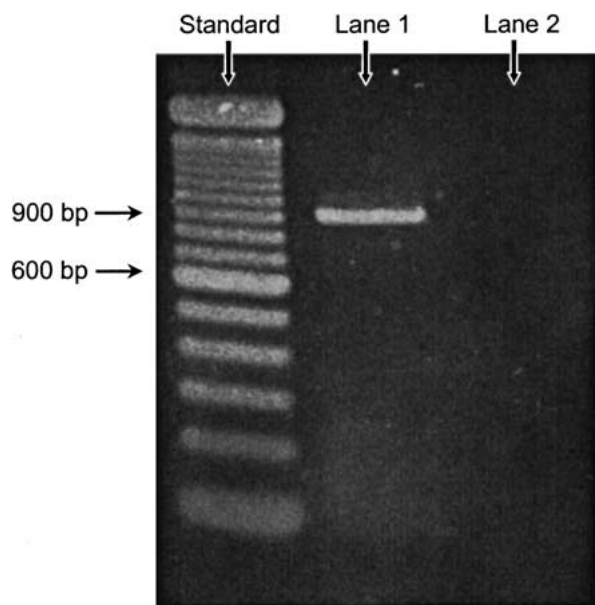


Fig. 2. HUVECs express the mineralocorticoid receptor mRNA. *Standard:* 100 bp ladder. *Lane 1:* 900 bp fragment of human mineralocorticoid receptor, amplified from cDNA synthesized from total RNA of HUVECs. *Lane 2:* Negative control (no reverse transcriptase added to cDNA synthesis mixture). RNA was treated with DNase I prior to cDNA synthesis.

RT-PCR clearly shows that mRNA is expressed in the preparation we use.

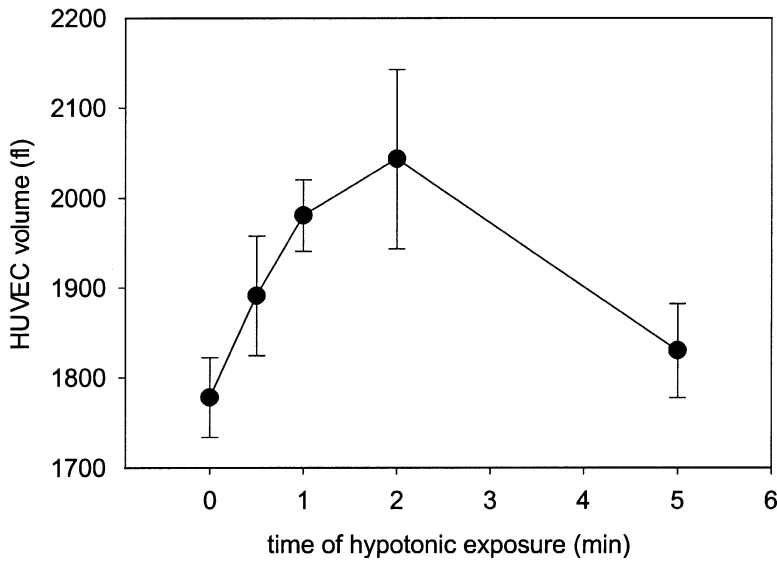


Fig. 3. AFM measurements on HUVECs exposed to hypotonicity. HUVEC volume transiently changes in response to a 20% dilution of the culture medium. HUVECs regulate their volume within 5 minutes (mean data of 10 measurements, \pm SEM).

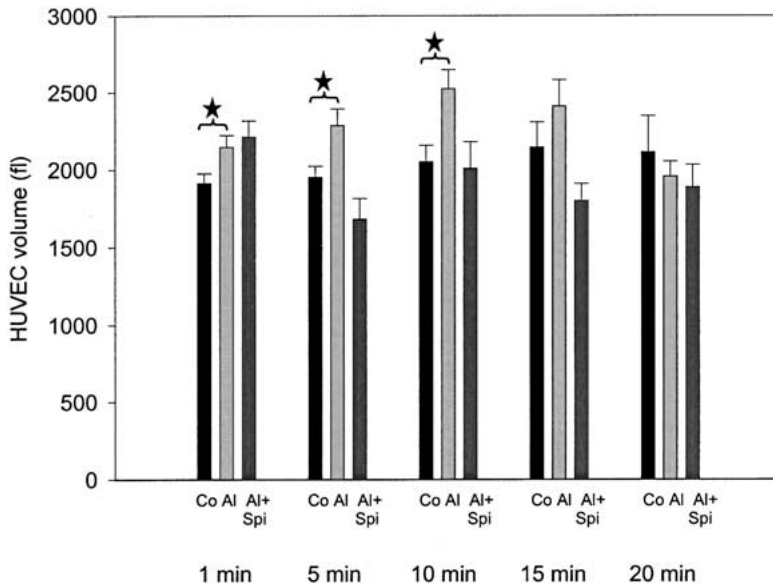


Fig. 4. AFM measurements on HUVECs exposed to solvent (*Co*), aldosterone (*Al*) or aldosterone plus spironolactone (*Al + Spi*). HUVEC volume is measured over a time scale from 1 to 20 minutes. Asterisks indicate significant difference between respective mean data. For further details see Table 1.

Another prerequisite for our study is the detection of cell volume by AFM. We assumed that reduction of extracellular osmolarity by about 20% should significantly swell HUVECs. This experimental procedure was used to test the method of volume determination by AFM. Cells were cultured, exposed to the hypotonic stimulus and then fixed at different time intervals. Total volume of the HUVEC monolayer was determined, and mean HUVEC volume calculated. Figure 3 shows the change in volume in response to hypotonicity. Cell swelling is visible already 30 s after the stimulus, increasing to a maximum after 2 minutes and decreasing thereafter. Since this pattern of behavior was expected for volume-regulated cells we proceeded with experiments applying aldosterone. We cultured HUVECs on glass

until they reached confluence and then exposed them to aldosterone. We measured cell volume 1, 5, 10, 15 and 20 minutes after hormone addition. We applied an aldosterone concentration of 100 nmol/l. As indicated in Fig. 4 and summarized in Table 1, we found a transient increase in cell volume that was detectable 1 minute after hormone application. It increased to a maximum 10 minutes after addition of aldosterone and then returned to control values 10 minutes later. 10 minutes of pre-incubation with the receptor antagonist spironolactone prevented the late aldosterone responses measured after 5 and 10 min, but not the immediate response measured after 1 min. Accompanying experiments where only the solvent, ethanol (0.1%), was added, served as control experiments.

Table 1. Aldosterone-induced volume changes in HUVECs

Time	Volume of HUVECs (femtoliter)				
	Control	Aldosterone	Aldosterone + Spironolactone	Aldosterone + Amiloride	Aldosterone + Cariporide
1 min	1915 ± 60 (16)	2146 ± 76* (16)	2212 ± 103 ^{ns/sp} (16)	2145 ± 64 ^{ns/am} (9)	2352 ± 89 ^{ns/car} (9)
5 min	1953 ± 70 (7)	2287 ± 106* (8)	1684 ± 132 [§] (7)	1691 ± 88 [#] (9)	2315 ± 114 ^{ns/car} (6)
10 min	2053 ± 105 (10)	2526 ± 122* (11)	2010 ± 170 [§] (4)	1834 ± 102 [#] (9)	2603 ± 90 ^{ns/car} (6)
15 min	2144 ± 165 (7)	2412 ± 170 ^{ns/co} (7)	1800 ± 111 [§] (4)	2031 ± 81 [#] (4)	2681 ± 120 ^{ns/car} (6)
20 min	2113 ± 233 (4)	1956 ± 99 ^{ns/co} (4)	1888 ± 146 ^{ns/sp} (4)	1863 ± 97 ^{ns/am} (4)	2101 ± 134 ^{ns/car} (6)

*Aldosterone versus control ($P < 0.05$).

^{ns/co}Aldosterone versus control (not significant).

[§]Aldosterone plus spironolactone versus aldosterone ($P < 0.01$).

^{ns/sp}Aldosterone plus spironolactone versus aldosterone (not significant).

^{ns/car}Aldosterone plus cariporide versus aldosterone (not significant).

[#]Aldosterone plus amiloride versus aldosterone ($P < 0.01$).

^{ns/am}Aldosterone plus amiloride versus aldosterone (not significant).

In parentheses, n = number of samples (10 single HUVECS per sample).

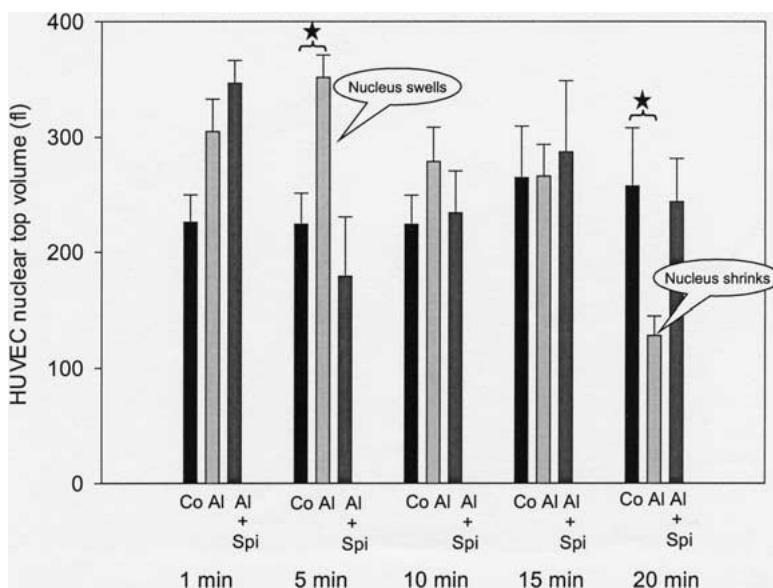


Fig. 5. AFM measurements on HUVECs exposed to solvent (Co), aldosterone (Al) or aldosterone plus spironolactone (Al + Spi). The nuclear top part (section line at half height) of HUVEC volume is measured over a time scale from 1 to 20 minutes. The upper half “nuclear top volume” of HUVECs mainly represents the nuclear compartment. Strong nuclear swelling after 5 minutes and strong nuclear shrinkage after 20 minutes of aldosterone exposure are emphasized. For further details see Table 2. Asterisks indicate significant difference between respective mean data.

In addition, we performed experiments along the same time scale where the drugs (spironolactone, amiloride or cariporide) were applied in the absence of aldosterone. (*data not shown*). We could not detect any statistically significant changes in cell volume in response to the individual inhibitors. However, we observed a tendency of a volume decrease when spironolactone or, in particular, amiloride was applied. Due to the rather large scatter of the data (heterogeneity in individual cell volume), the drug effects in absence of aldosterone were not statistically significant.

In order to focus on the nuclear compartment we electronically sectioned the cells and analyzed the top half of the HUVECs. We assumed (see Fig. 1) that the cell top is mainly composed of nucleoplasm and, only to a minor extent, of cytosol. The analysis of the

cell tops is shown in Fig. 5 and Table 2. The results are striking: There is an immediate (1 min after aldosterone) hormone response, indicating nuclear swelling. It is a transient change with a maximum at 5 min after aldosterone, followed by volume relaxation. Then, 20 min after aldosterone, we observe a sharp shrinking of the nuclear compartment. The specificity of the response was investigated by another series of experiments where spironolactone was applied 10 min before aldosterone was added. In agreement with the change in total cell volume described above, spironolactone did not affect immediate (1 min) nuclear swelling but completely blocked the late (5 to 20 min) swelling/shrinking processes of the nuclear compartment.

Cell volume changes are usually accompanied by regulatory processes mediated by plasma membrane

Table 2. Aldosterone-induced volume changes of the nuclear compartment

Time	Nuclear compartment of HUVECs (femtoliter)		
	Control	Aldosterone	Aldosterone + Spironolactone
1 min	226 ± 23 (16)	304 ± 28* (16)	347 ± 19 ^{ns/sp} (16)
5 min	224 ± 26 (7)	352 ± 19** (8)	179 ± 51 [§] (7)
10 min	224 ± 25 (10)	279 ± 29 ^{ns/co} (11)	234 ± 36 ^{ns/sp} (4)
15 min	265 ± 44 (7)	266 ± 27 ^{ns/co} (7)	287 ± 61 ^{ns/sp} (4)
20 min	257 ± 50 (4)	128 ± 16** (4)	244 ± 37 [§] (4)

*Aldosterone versus control ($P < 0.05$).

**Aldosterone versus control ($P < 0.01$).

^{ns/co}Aldosterone versus control (not significant).

[§]Aldosterone plus spironolactone versus aldosterone ($P < 0.01$).

^{ns/sp}Aldosterone plus spironolactone versus aldosterone (not significant).

In parentheses, n = number of samples (10 single HUVECS per sample).

transporters. Assuming that Na^+/H^+ exchange could mediate aldosterone-induced salt and water movement across the plasma membrane, we performed a similar series of experiments as done with spironolactone. The mean data are summarized in Table 1. The Na^+/H^+ exchange blocker cariporide was added 10 minutes prior to aldosterone treatment. Surprisingly, this blocker was ineffective at a concentration (10 $\mu\text{mol/l}$) that should inhibit the antiporter (if expressed and functional). In another series of experiments we applied amiloride using concentrations (1 $\mu\text{mol/l}$) that block epithelial Na^+ channels. Like cariporide and spironolactone, amiloride could not prevent immediate aldosterone-induced swelling. However, the drug successfully prevented any changes in cell volume that occurred between 5 min and 20 min after aldosterone application.

Discussion

We currently observe a paradigm shift concerning the role of aldosterone in mediating physiological and pathophysiological processes in humans. There are at least four new perspectives that should be considered: (i) Aldosterone not only acts on classical organ structures like renal epithelium but also on the cardiovascular system, including fibroblasts (Weber & Brilla, 1991), cardiomyocytes (Silvestre et al., 1998), vascular smooth muscle cells (Meyer & Nichols, 1981) and endothelial cells (Rajagopalan et al., 2002). (ii) The site of aldosterone synthesis is not limited to the adrenal cortex, but also occurs at extra-adrenal sites, including vascular smooth muscle cells and endothelium (Hatakeyama et al., 1994; Silvestre et al., 1998). It has been proposed that aldosterone produced in the vascular endothelium may act on vascular smooth muscle cells, binding to the classical mineralocorticoid receptor, thereby acting in a paracrine manner (Duprez et al., 2000). (iii) Besides the classical genomic response mediated by intracel-

lular mineralocorticoid receptors there is clear evidence for fast pre-genomic actions of the hormone in various cell types and at different cellular levels. Such responses can occur in seconds to minutes and involve plasma membrane ion channels/transporters (Wehling, Kasmayr & Theisen, 1989), intracellular enzymes (Doolan, O'Sullivan & Harvey, 1998) and messengers such as protons (Oberleithner et al., 1987) or Ca^{2+} (Gekle et al., 1996). (iv) Aldosterone has a number of deleterious effects on the cardiovascular system, including myocardial fibrosis, vascular injury and endothelial dysfunction (Rajagopalan et al., 2002; Miric et al., 2001). In kidney, aldosterone may promote fibrosis and thus mediate progressive renal dysfunction (Epstein, 2001).

Some years ago we observed in living aortic endothelial cells that aldosterone transiently increased cell volume (Schneider et al., 1997b). The aldosterone response occurred in minutes and could be inhibited by amiloride. Since AFM was applied in this previous study, it was possible to analyze the 3-D morphology of the adherent endothelial cells together with cell volume. Although AFM images of living cells have only poor resolution, it became obvious in a later analysis (Oberleithner et al., 2000) that the volume change mainly occurred at the site of the cell nucleus. Based on this data we postulated that volume "cycles" between intracellular compartments, a cycling induced by aldosterone. In order to shift volume from the cytosol to the nucleoplasm, osmotic driving forces are necessary. Since the nuclear envelope is usually highly permeable to ions but selective for macromolecules, we focused on hormone-induced macromolecule transport in a model system. We observed in aldosterone-injected oocytes of *Xenopus laevis* that macromolecule shifts occur between cytosol and nucleoplasm in response to the hormone (Schafer et al., 2002). Most likely, volume shifts were mediated by receptor import into the cell nucleus and export of transcribed mRNA into the cytosol. These observed macromolecule shifts could explain changes

in cell volume. The aim of the present study was to test this hypothesis in endothelial cells. This time, we chose a protocol applying AFM to fixed cells. Fixation stiffens the cell and thus greatly improves the images. We fixed the cells in the incubator at physiological conditions on a predefined time scale. Scanning was performed in buffered solution. Cells could be maintained over days in fluid (4°C) without changing shape or volume. Experiments using hypotonic treatment indicate that this approach gives reliable results. HUVECs change volume in response to hypotonicity and perform a volume-regulatory decrease. The experimental scatter of the unpaired experiments was kept low by taking care of the following parameters: (i) HUVECs were cultured and fixed at identical conditions except for one specific treatment (e.g., aldosterone addition). (ii) AFM was performed at technically comparable conditions including scan size, scan force, scan frequency, etc. When these parameters are kept constant, the AFM volume measurements are easy to perform and reliable. A $100 \times 100 \mu\text{m}^2$ HUVEC monolayer including about 10 individual cells is scanned and analyzed for cell volume in about 2 minutes. The unique advantage of measuring cell volume by AFM is that volume can be analyzed independently of cell shape. Thus, cell morphology can be analyzed in parallel with cell volume.

The changes of total cell volume in response to aldosterone are remarkable and must be considered step by step. The onset is immediate (within one minute) and independent of the classical receptors, since spironolactone was ineffective. This is typical for a pre-genomic response. It smoothly intercalates with the genomic response indicated by sensitivity to spironolactone 5 min after hormone exposure. Noteworthy is the transient nature of the volume change and the biphasic response observed in the nuclear compartment. The sharp volume increase of the cell top (nuclear compartment) at the onset of the hormone and the sharp decrease about 15 to 20 min later strongly indicate that the volume change occurs in this nuclear compartment. It matches the previous observations of receptor import into the nucleus and mRNA export into the opposite direction after a delay period of about 20 min (Schafer et al., 2002). Our own RT-PCR measurements and inhibition by spironolactone support the view that (i) HUVECs express mineralocorticoid receptors (Brown et al., 2000), (ii) receptor translocation from the cytosol into the nucleoplasm can be blocked by receptor antagonists, (iii) mineralocorticoid receptor antagonists not only act on kidney but also on vascular endothelium. We propose a model (Fig. 6) where the aldosterone-induced initial nuclear swelling is indicative for receptor import based on previous observations made in the oocyte system (Schafer et al., 2002). Similarly, the late nuclear shrinkage about 20 min after aldosterone

exposure is expected to occur when osmotically active macromolecules, such as mRNA complexed to proteins, leave the nucleus. This has been demonstrated at the single-molecule level previously (Schafer et al., 2002).

To our surprise, cariporide, a potent inhibitor of subtype-1 Na^+/H^+ exchange (Symons & Schaefer, 2001), did not block the volume changes. Usually, the antiporter mediates volume regulation whenever a cell needs to gain volume (Hayashi, Szasz & Grinstein, 2002). This situation happens at the onset of aldosterone action when receptors, accompanied by water, move into the nucleus and thus shrink the cytosolic compartment. In contrast, a low dose of amiloride wipes out the aldosterone-induced late response, indicating that epithelial Na^+ channels do mediate the volume changes across plasma membrane. Indeed, epithelial Na^+ channels have been shown to exist in vascular endothelial cells (Golestaneh et al., 2001). They are regulated by aldosterone and require an intact cytoskeleton. The mechanism is in agreement with a previous study that shows up-regulation of cell volume-sensitive Na^+ channels induced by glucocorticoids (Gamper et al., 2000). The data are also consistent with results obtained in rat distal nephron where spironolactone strongly decreased Na^+ -channel abundance (Nielsen et al., 2002). At least in kidney, there is no doubt that a functional epithelial Na^+ channel has a major impact on the regulation of extracellular volume and blood pressure (Stokes, 1999; Frindt et al., 2002), while the role of Na^+ channels in endothelial cells is less clear.

It is interesting to note that the immediate onset of aldosterone-induced endothelial cell swelling is independent of the activity of Na^+ channels. This mechanism is still unknown. It could be based on an immediate calcium increase observed recently in endothelial cells (Schneider et al., 1997a). Such a rise in intracellular calcium could destabilize supramolecular structures (e.g., F-actin depolymerization) and thus lead to osmotic water uptake by the cell.

MEDICAL IMPLICATIONS

The present study shows for the first time a rapid response in endothelial cell volume induced by aldosterone and inhibited by two different mechanisms: (i) by a block of plasma membrane epithelial Na^+ channels using amiloride (pre-genomic blockade) and (ii) by a block of intracellular receptors using spironolactone (genomic blockade). Both experimental strategies could be useful in clinical applications. Aldosterone-induced swelling of endothelial cells will not affect blood flow in large blood vessels, including veins and arteries. However, endothelial swelling could affect blood flow in capillaries. With capillary

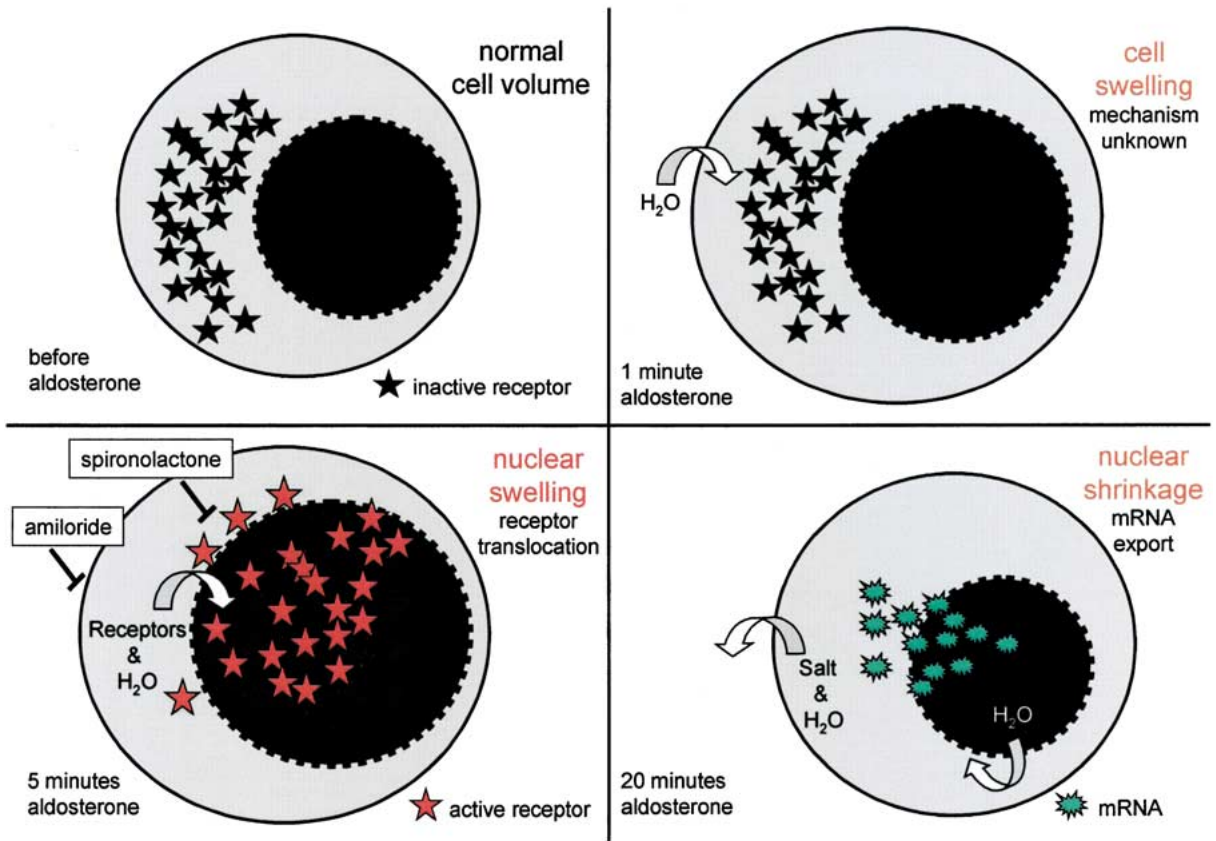


Fig. 6. Model of aldosterone action in HUVECs. *Before aldosterone:* Cell with intracellular aldosterone receptors. *1 minute of aldosterone (immediate response):* Upon aldosterone stimulation water enters the cytosol driven by yet unknown mechanisms. The cell swells. *5 minutes of aldosterone:* Activated intracellular mineralocorticoid receptors translocate into the nucleus. Water move-

ment occurs due to oncotic driving forces. The nucleus swells and transcription is initiated. Swelling is prevented by amiloride or spironolactone. *20 minutes of aldosterone (late response):* Aldosterone-induced mRNA is exported. Water moves from the nucleoplasm into the cytosol due to oncotic driving forces. Salt and water exit the cell due to volume regulation.

diameters in the range of about 5 to 10 μm , a 10% swelling of the endothelium, induced by aldosterone, could affect capillary blood flow by almost 50%. This is due to the fact that, according to Hagen-Poiseuille's law, capillary radius and blood flow correlate to the 4th potency. Increased red blood cell shear stress in narrow capillaries could induce von-Willebrand factor exocytosis in endothelial cells (Goerge et al., 2002). Such pro-coagulatory mechanisms could lead to cardiovascular injuries (Stier, Jr. et al. 2002) if not prevented by spironolactone, amiloride or their analogs.

Another field of interest is cell growth and cell metabolism regulated by aldosterone. It has been shown that endogenous cardiac aldosterone synthesis may cause cardiac hypertrophy (Takeda et al., 2000). Furthermore, mineralocorticoid receptor antagonism improves endothelial function in early atherosclerosis (Rajagopalan et al., 2002).

In our experiments we applied concentrations of aldosterone about 100 times higher than usually present in plasma. However, tissue concentrations of

aldosterone are about 20 times higher than plasma concentrations (Lijnen & Petrov, 2000) and we expect even higher local concentrations if aldosterone is used in a paracrine or autocrine manner. It is a matter of speculation whether aldosterone could serve as an endothelial mitogen during organ ontogeny similarly as described for an endothelial growth factor, the so-called hepatoma-derived growth factor HDGF, which was shown to drive nephrogenesis (Oliver & Al Awqati, 1998).

Cultured endothelial cells of bovine aorta transiently swell within minutes when challenged with very low concentrations (0.1 nM) of aldosterone (Schneider et al., 1997b). In HUVECs we needed much higher concentrations to elicit a similar response. Since we cannot explain this apparent discrepancy, we can only speculate: We assume that high aldosterone concentrations occur locally by paracrine secretion in vivo, in combination with a slow blood flow that allows the formation of unstirred layers at the endothelial cell surface. Slow blood flow occurs in umbilical veins but not in the aorta. The local aldosterone concentration

in the intact tissue could thus define the sensitivity of the system. A high hormone concentration in vivo could desensitize the target cell and vice versa. Therefore, future research should focus on the analysis of local hormone concentrations rather than on measurements of systemic concentrations.

From the biochemical point of view we interpret transient cell-volume changes induced by steroids as transcriptional/translational processes in the cell. From the physiological point of view we explain the cell volume change as the result of intracellular macromolecule shifts accompanied by volume-regulatory mechanisms. From the medical point of view we propose a pro-coagulatory role for aldosterone. Whatever view is taken, aldosterone action in endothelial cells can be blocked either at the pre-genomic level (block of Na⁺ channels by amiloride) or at the genomic level (block of intracellular receptors by spironolactone). This could help prevent vasculopathies caused by aldosterone.

We thank Mrs. Marianne Wilhelmi and Hannelore Arnold for their excellent HUVEC culture work. We thank Drs. Kleemann and Lang (Aventis Pharma GmbH, Deutschland) for kindly providing cariporide. The study was supported by IZKF Münster (Project A9) and the Volkswagenstiftung (Project BD 151103).

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