Plasma sodium stiffens vascular endothelium and reduces nitric oxide release

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Dietary salt plays a major role in the regulation of blood pressure, and the mineralocorticoid hormone aldosterone controls salt homeostasis and extracellular volume. Recent observations suggest that a small increase in plasma sodium concentration may contribute to the pressor response of dietary salt. Because endothelial cells are (*i*) sensitive to aldosterone, (*ii*) in physical contact with plasma sodium, and (*iii*) crucial regulators of vascular tone, we tested whether acute changes in plasma sodium concentration, within the physiological range, can alter the physical properties of endothelial cells. The tip of an atomic force microscope was used as a nanosensor to measure stiffness of living endothelial cells incubated for 3 days in a culture medium containing aldosterone at a physiological concentration (0.45 nM). Endothelial cell stiffness was unaffected by acute changes in sodium concentration <135 mM but rose steeply between 135 and 145 mM. The increase in stiffness occurred within minutes. Lack of aldosterone in the culture medium or treatment with the epithelial sodium channel inhibitor amiloride prevented this response. Nitric oxide formation was found down-regulated in cells cultured in aldosterone-containing high sodium medium. The results suggest that changes in plasma sodium concentration *per se* may affect endothelial function and thus control vascular tone.

atomic force microscopy | cell stiffness | endothelial dysfunction | eplerenone

Essential hypertension is the sum of interactions between multiple environmental and genetic factors (1, 2). The role of dietary salt in causing essential hypertension and other harmful cardiovascular effects, independent of blood pressure, derives from epidemiological studies, experimental models (particularly in primates), physiological and biochemical studies, controlled clinical trials, and genetic and mortality studies (3). Humans exposed to a high-salt diet may develop hypertension (4). In such individuals, the kidney has a limited ability to excrete the daily uptake of sodium and tends to retain the salt, most likely osmotically inactive, in skin and other extracellular compartments (5). This internal sodium "escape" buffer, which is probably too small in humans with high blood pressure, indicates that extrarenal sodium balance plays an important role in blood pressure control (6). Salt and water balance is regulated by a multitude of factors/mediators, of which aldosterone is one of them. This mineralocorticoid hormone is known to control the activity of epithelial sodium channels (ENaC) in the renal collecting duct. It also acts on ENaC in endothelia (7, 8), where it may cause the cell to swell (9, 10), stiffen (11, 12), and alter its output of nitric oxide (13–15).

Little is known about how dietary salt raises blood pressure. Salt consumption increases thirst and the uptake of fluid. As a consequence, there is a transient increase in plasma volume and a subsequent rise in arterial blood pressure as the extracellular volume returns to normal (16). Obviously, the organism tries to regain the original extracellular volume (e.g., by reducing the volume by vascular smooth muscle contraction) at the expense of an increased arterial blood pressure. This view is in agreement with the fact that diuretics lower blood pressure. However, this well known effect of diuretics cannot be explained exclusively by a decrease in extracellular volume. Other components of the extracellular fluid, as e.g., electrolyte concentrations, could be important determinants.

Recently, plasma sodium has been suggested to play a primary role in the control of blood pressure because a small increase in plasma sodium (1–3 mM) was found in individuals with hypertension (17, 18). This observation triggered the idea that the vascular endothelium could participate in a sodium-mediated blood vessel function. Endothelial cells express ENaC in response to aldosterone. This sodium-selective ion channel could act as a functional link between the plasma and the endothelial cell. For instance, the sodium channel blocker amiloride prevents aldosterone-induced cell swelling (10) and cell stiffening (15), which suggests that apical plasma membrane sodium influx controls the volume and stiffness of endothelial cells. High endothelial deformability is a prerequisite for normal endothelial function, including shear stress-induced release of nitric oxide (19).

We have tested the proposal that endothelial stiffness and deformability is influenced by small changes in plasma sodium concentration. Atomic force microscopy (AFM), a nanotechnique that measures stiffness and deformability of living endothelial cells, was used (20). The AFM tip was used as a mechanical sensor to quantify these properties in individual endothelial cells exposed to increasing sodium concentrations. In the absence of aldosterone, i.e., at nonphysiological conditions, the stiffness and deformability of the cells were unchanged by alterations of extracellular sodium, but in the presence of aldosterone, i.e., at physiological conditions, the cells became increasingly affected by sodium concentrations >135 mM. This finding suggests that plasma sodium concentration determines endothelial cell elasticity, an important parameter in the control of blood pressure.

Results

Endothelial Stiffness Depends on Sodium and Aldosterone. Before the experiments, endothelial cells were kept in two experimental conditions. One set of cells was maintained in an aldosterone-free culture medium to which eplerenone was added to prevent any effects of endogenous aldosterone. A second set of cells was maintained for 3 days in aldosterone-containing medium. Endothelial cell stiffness was continuously measured (one measurement per second = 1 Hz) as the extracellular sodium concentration was increased stepwise from 120 to 160 mM. Fig. 1 shows a representative measurement performed in cells treated with aldosterone. When sodium was raised in the superfusate from 125 to 135 mM, the slope of the force-distance curve remained virtually constant. In contrast, when sodium was further increased to 145 mM the slope

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Abbreviations: ENaC, epithelial sodium channels; AFM, atomic force microscopy.

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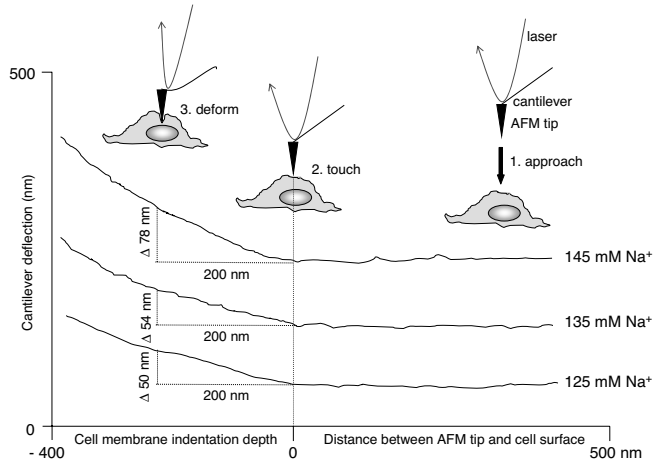

Fig. 1. Force–distance curves obtained in a single endothelial cell exposed subsequently to increasing sodium concentrations (exposure time to one individual sodium concentration was ≈3 min). Each curve was obtained in ≈1 s and repeated 5 to 10 times. The slopes of the curves were analyzed from the cantilever deflections related to the indentation depth.

steepened significantly. The results of the two series of experiments (i.e., eplerenone-treated cells and aldosterone-treated cells) are displayed in Fig. 2. There is one important difference between the results obtained in the two sets of experiments: Aldosterone treatment rendered endothelial cell stiffness sensitive to extracellular sodium. It is most interesting that the sodium sensitivity was only apparent at sodium concentrations in the physiological range and above. In cells, treated with aldosterone, increasing sodium concentration from 135 to 145 mM led to a sharp rise in cell stiffness (22.4%; $P < 0.001$). Changes in cell stiffness occurred within minutes and were apparently not transient in nature. The reversibility of the changes in cell stiffness was tested in paired experiments, increasing sodium concentration first from 140 to 145 mM and then, a few minutes later, decreasing sodium from 145 to 140 mM. In five experiments (i.e., in five individual endothelial cells) cell stiffness increased by 10.5 ± 1.9% when sodium was raised by 5 mM and then decreased by 9.9 ± 1.7% when sodium was lowered to the original value (140 mM).

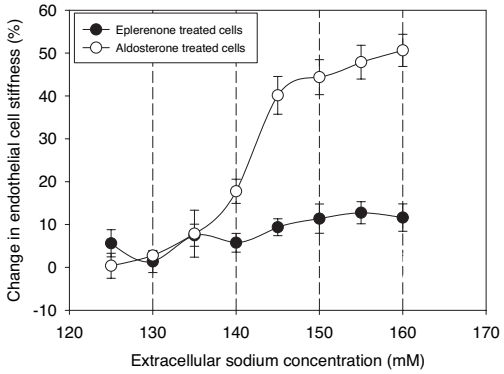

Fig. 2. Changes in endothelial cell stiffness in response to increasing sodium concentration measured in buffered electrolyte solution (constant osmolality). Sodium concentration was varied between 120 and 160 mM. Osmolality was kept constant by adequate additions of mannitol. Measurements were started with 120 mM sodium in the solution (reference solution). Single cells in the monolayer were chosen and force curves were obtained. Then, the reference solution was exchanged stepwise for solutions containing increasing sodium concentrations (exposure time to one individual salt concentration was ≈3 min). The stiffness measurement obtained in cells bathed with 120 mM sodium served as the reference value and deviations from this value (in %) are reported here. Mean values of 10 independent measurements per series of experiments are given ± SEM. In aldosterone-treated endothelium, mean values of 140 mM sodium and higher are significantly different compared with mean values measured at 120 mM sodium ($P < 0.01$). Also, comparisons of mean values between eplerenone-treated endothelium and aldosterone-treated endothelium, obtained at the same sodium concentrations, reveal statistical significances ($P < 0.01$) at 140 mM sodium and higher.

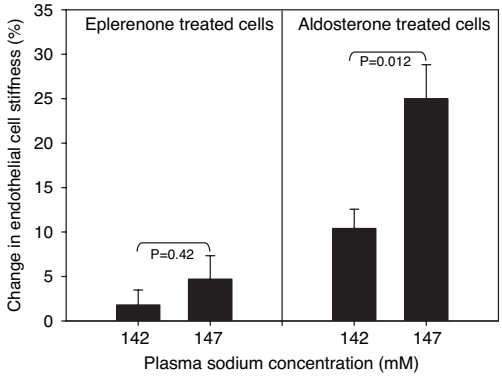

Fig. 3. Changes in endothelial cell stiffness in response to increasing sodium concentration in human plasma. Blood was drawn from volunteers in heparin-coated syringes and centrifuged, and then plasma sodium was analyzed. Plasma with a sodium concentration of 137 mM was used as a reference (mean data given as deviation from this value in %). NaCl was added to achieve concentrations of 142 and 147 mM sodium. Monolayers were exposed subsequently to the three different plasma samples and stiffness was measured in individual cells under all three conditions. Mean values of 10 independent measurements are given ± SEM. In eplerenone-treated endothelium cell stiffness did not change significantly in high sodium (147 mM) compared with a sodium concentration of 142 mM but increased in aldosterone-treated cells.

In contrast, eplerenone-treated cells subjected to an increase in sodium concentration from 135 to 145 mM showed no statistically significant change. Therefore, extracellular sodium controls cell stiffness only in the aldosterone-treated endothelium.

In should be mentioned that the baseline cell stiffness differed between eplerenone-treated and aldosterone-treated cells. Aldosterone-treated cells cultured in normal medium exhibited a stiffness ≈78% greater than that of control cells (11). In this account we focus on the changes in cell stiffness rather than on the absolute values. Nevertheless, it should be kept in mind that any change of stiffness caused by an increase in sodium concentration was additional to the baseline stiffness value. The results indicate that a cell pretreated with aldosterone and acutely exposed to high sodium (150 mM) is ≈120% stiffer than a cell cultured in the absence of aldosterone.

High Sodium in Human Plasma Stiffens Endothelial Cells. To imitate physiological conditions, blood samples were taken from volunteers, and the plasma with its endogenous sodium concentration (137 mM) was used as the reference plasma. To this plasma, we added NaCl to increase sodium to 142 and 147 mM. Endothelial cells were then superfused with the plasma samples, starting with the reference plasma (137 mM sodium). As summarized in Fig. 3, stiffness of eplerenone-treated cells was not significantly altered when plasma sodium was increased from 142 to 147 mM (2.9%; $P = 0.42$). In contrast, cells that had been treated with aldosterone for 3 days stiffened significantly (14.6%; $P = 0.01$).

Amiloride Prevents Sodium-Induced Endothelial Stiffening. Similar to kidney tubules, endothelia also express ENaC (8, 11), and aldosterone that modulates the insertion of channels into the apical membrane of distal tubule cells is its major regulator (9). Results obtained in long-term experiments indicate that, after the exposure to aldosterone, endothelial cells swell (9), stiffen, and simulta-

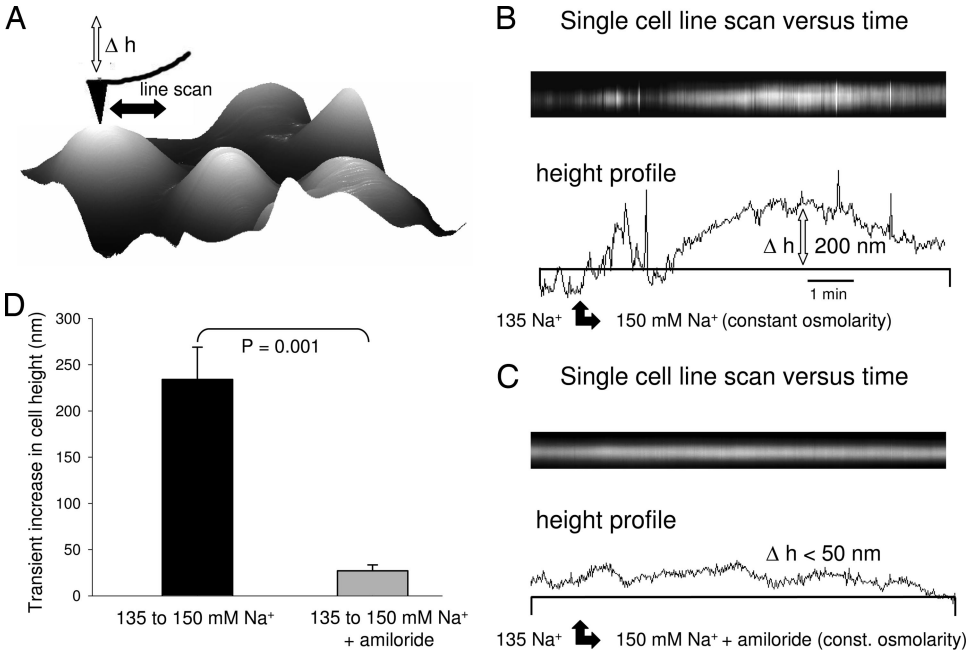

Fig. 4. Surface line scans versus time in endothelial cells using AFM. (*A*) Schematic showing living endothelial cells and the AFM tip engaged to the apical membrane surface. The AFM tip scans at 1 Hz (temporal resolution 1 s) along one line (lateral resolution ≈100 nm) forth and back in horizontal direction (*x* axis). (*B*) Line scan of a single endothelial cell. The profile shows the transient increase in cell height (h) when the sodium concentration in the buffer is increased from 135 to 150 mM sodium. (*C*) As described in *B* except that amiloride (1 μM) was present in the high sodium solution. (*D*) Mean data (± SEM; $n = 15$) of the transient increases of cell heights.

neously increase their plasma membrane surface (11). In the present study, we tested whether inhibition of sodium channels influences sodium-dependent stiffness of endothelial cells. Amiloride, a potassium-sparing diuretic that blocks sodium channels, was added to aldosterone-treated endothelium during superfusion with low (135 mM) and, subsequently, high (150 mM) sodium. Amiloride was ineffective at a low sodium concentration (increase in stiffness at 135 mM sodium: 2.4 ± 4.05% compared with 6.4 ± 4.46%; $n = 10$; $P = 0.46$). In contrast, amiloride was highly effective at a high sodium concentration (increase in stiffness at 150 mM sodium: 52.6 ± 11.38% versus 16.8 ± 7.05%; $n = 10$; $P = 0.003$). In control cells (no aldosterone treatment before the experiments), amiloride had no effect on cell stiffness.

Amiloride Prevents Sodium-Induced Transient Cell Volume Increase. Sodium influx into cells is expected to increase cell volume because net addition of solutes is accompanied by water movement. We tested this by measuring the cell height of endothelial cells by using the line scan mode (i.e., repetitive scans in the same *x* axis, *y* axis = time). This method is explained in Fig. 4*A*. As indicated in Fig. 4*B*, an increase of the superfusate sodium concentration from 135 to 150 mM (at constant osmolality; 30 mM mannitol was added to the former solution to compensate for the lack of osmolality) induced a transient change in cell height of ≈100–300 nm. The cell height returned to the initial control value within a few minutes after the rise in sodium, most likely because of regulatory mechanisms (21). The height increase was ≈5% of total endothelial cell height (≈ 4 μm) and corresponded to a similar change in cell volume. Such small changes could be detected only by the AFM technique because of its high resolution in the *z* axis. Fig. 4*C* shows that the transient sodium-induced increase in cell volume could be prevented by amiloride added to the high sodium superfusate. This result indicates that sodium influx through sodium channels caused the change in cell volume. Fig. 4*D* summarizes the results of the line scan measurements. The data show that endothelial cells, pretreated with aldosterone, swell in response to an increase in sodium concentration, and the sodium channel blocker amiloride blocks this effect.

High Ambient Sodium Reduces Endothelial Deformability. We also tested the effect of changes in sodium concentration on endothelial deformability in aldosterone-treated cells by applying constant mechanical force to the apical cell surface during cell imaging.

Cells exposed to low sodium are more deformable by scan forces than the same cells exposed to high sodium as demonstrated in the two AFM images of Fig. 5. Endothelial cells scanned with constant force and constant frequency in a solution of 135 mM sodium are visibly flattened by the scanning AFM tip. In contrast, in medium containing 150 mM sodium the same endothelial cells (paired experiment) resisted the "pressure" of the scanning AFM tip and remained prominent.

Nitric Oxide Formation of Aldosterone-Exposed Cells Is Down-Regulated in High Sodium Medium. For this series of experiments, GM7373 cells were exposed to 135 and 150 mM sodium-containing medium in the absence or presence of 0.45 mM aldosterone. To simulate shear stress, the culture flasks were kept on a shaker that rhythmically tilted a thin layer of medium across the apical cell surface. In the harvested culture medium there was a significant decrease of nitrite concentration in the high sodium medium, indicating down-regulation of nitric oxide formation (Fig. 6). This down-regulation could be observed only in the aldosterone-treated cells. In the absence of aldosterone, the cells were insensitive to changes in ambient sodium. These results indicate that there is a functional link between extracellular sodium and nitric oxide metabolism.

Discussion

In comparison with the diet habitually ingested by preagricultural *Homo sapiens* living in the Stone Age, the diet of contemporary *H. sapiens* contains ≈10 times more sodium (22). Before the ingested sodium is eliminated by the kidneys, it will pass the extracellular

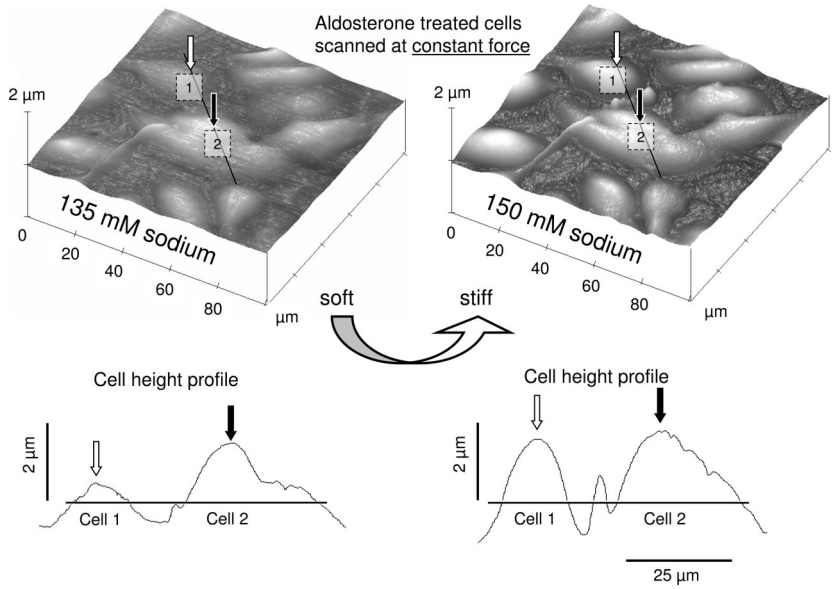

Fig. 5. AFM images of living (aldosteroen-treated) endothelial cells and their respective profiles. An endothelial cell monolayer was scanned with constant force (5 nN) and constant frequency (1 Hz) at two different sodium concentrations in the bath, 135 mM sodium (*Left*) and 150 mM sodium (*Right*). Lines and arrows indicate the location of the profiles shown below the images. At low sodium, cells are flattened by the force of the AFM stylus applied to the endothelial cells. At high sodium, cells resist the applied forces and thus appear prominent.

compartments, including the vascular system. Our results show that an acute increase of plasma sodium concentration within the narrow range observed in Western populations on high-salt diet (18) can alter the mechanical properties of the vascular endothelium. Endothelial cells, however, respond to sodium only when grown in culture medium enriched with aldosterone, which is known to retain sodium within the extracellular compartments.

Several lines of evidence indicate that aldosterone not only acts on kidney but also on the cardiovascular system (23). In the kidney, the hormone plays a major role in adjusting sodium and potassium transport in collecting ducts to the metabolic requirements. Mild hyperaldosteronism, a metabolic disorder found in >10% of hypertensive humans, causes sodium retention combined with hypokalemia (24). In contrast to the kidney, where the cellular and molecular actions of aldosterone are well defined (25), there is less information on how the steroid acts on blood vessels. Recent studies have shown that, similar to renal tubules, aldosterone activates epithelial sodium channels in endothelial cells (8, 10, 11), suggesting a possible role in the control of blood pressure.

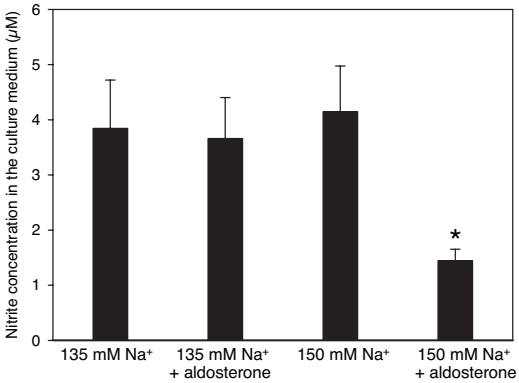

Fig. 6. Nitrite concentrations of different culture media after exposure to GM7373 bovine aortic endothelial cells. Culture flasks were mounted on a shaker in the incubator for mimicking shear forces. Aldosterone concentration = 0.45 nM. Three independent sets of experiments were performed (three nitrite measurements per set; number of single measurements per column = 9; ± SEM). The asterisk (150 mM sodium + aldosterone) indicates a significant difference of mean nitrite concentration in comparison with each of the other mean values ($P < 0.01$).

Plasma Sodium Determines Endothelial Stiffness. There is evidence that plasma sodium concentration *per se* may be a determining factor in the development of hypertension (18). Sodium accumulates in the extracellular space either when the kidneys cannot adequately adjust salt excretion to salt uptake and/or the concentration of aldosterone is raised. Salt can be stored, osmotically inactive, in the skin and other extracellular compartments (5), but the sodium storage capacity of such compartments is limited, particularly in hypertensive animals receiving mineralocorticoids (6). Thus, a limited ability to store sodium, a raised concentration of aldosterone, or a renal inadequacy to excrete sodium effectively may lead to an increase in plasma sodium concentration. A rise in plasma sodium has indeed been observed in hypertensive individuals (17). Such elevation of plasma sodium concentration is small (only a few mM) presumably because a rise in plasma osmolality is accompanied by water retention.

The results demonstrate that small acute changes in plasma sodium concentration have a significant effect on the stiffness and deformability of endothelial cells. As the variations in sodium concentration used in the present study were carefully balanced osmotically by addition of mannitol, the altered endothelial properties were not caused by shifts in osmolality.

Role of Epithelial Sodium Channels in Modulating Endothelial Stiffness. An important finding of the present experiments is the observed dependence of sodium-induced changes in endothelial function on aldosterone and sodium channels. Thus, amiloride, applied to aldosterone-treated endothelium, prevents the increase in stiffness that is elicited by a change of extracellular sodium from 135 to 150 mM. As demonstrated in Fig. 2, cell stiffness rises progressively with increments of extracellular sodium concentration but only when the endothelium has been treated with aldosterone. It is also of interest that the sodium-dependent effects are most pronounced in the narrow physiological range (between 135 and

145 mM). Here, we want to emphasize that what we refer to as "aldosterone treatment" is the more physiological condition when compared with culture conditions in which the hormone is absent. The aldosterone concentration used in our culture media is in the high normal range in the normal population (26).

The sodium-induced increase in cell stiffness is most likely linked to cell swelling because in aldosterone-treated endothelium amiloride reverses the increase in cell volume (9) and cell pressure (20). However, these data should be interpreted with particular care. In these two studies, aldosterone was applied over 3 days before the acute application of amiloride, and it is known that over such a long time period endothelial cells accumulate organic material, possibly aldosterone-induced proteins, and thus swell because of increased oncotic pressure (20). Nevertheless, acute aldosterone application to endothelial cells also leads to amiloride-inhibitable cell swelling, which is most likely caused by the uptake of sodium and water (27). When endothelial cell pressure (technically indistinguishable of cell stiffness) is measured in response to the acute administration of aldosterone with the same time scale (≈30 min), endothelial cells regulate their volume, while intracellular pressure increases (28). In addition, cells show signs of contraction. A comparable observation, i.e., cell contraction in response to the acute application of aldosterone, was made recently in renal epithelial cells (29).

The observation that aldosterone-induced cell swelling is blocked by amiloride suggests a link between endothelial cell stiffness and ENaC activity. It is presently not known how a change in intracellular sodium translates the extracellular signal (increase in sodium) into the cellular response (increase in stiffness). Recently, it has been discovered that intracellular sodium controls the open probability of epithelial sodium channels (30). Such a mechanism could limit sodium entry in the aldosterone-sensitive target cells (called sodium self-inhibition) and, at the same time, control cell stiffness.

Is a "Stiff" Endothelial Cell Functionally Inferior to a "Soft" One? The endothelium is a metabolically active "organ" able to release a number of vasodilator factors, including nitric oxide (31). Thus, mechanical stress causes nitric oxide synthase activation, leading to nitric oxide release and vasodilation (19). For example, large rhythmic deformations of the highly elastic endothelial cells *in vivo*, which are induced by shear stress, stimulate nitric oxide metabolism. In the present study, by mimicking a shear stress situation *in vitro*, we observed that high extracellular sodium concentration in conjunction with aldosterone stiffens endothelial cells and reduces nitric oxide release from the vascular endothelium. This could explain, at least in part, some of the cardiovascular disorders observed in hyperaldosteronism and hypoaldosteronism. For instance, Nishizaka *et al.* (32) demonstrated impaired endothelium-dependent flow-mediated vasodilation in hypertensive subjects with hyperaldosteronism, and Duffy *et al.* (15) showed that low-renin hypertension with relative aldosterone excess is associated with impaired nitric oxide-mediated vasodilation. In contrast, Orbach *et al.* (33) showed that in Addison's disease (chronic adrenal insufficiency) hyponatremia and hypotension were closely related to increased production of nitric oxide. Furthermore, there is evidence that salt loading down-regulates renal and vascular nitric oxide synthase expression (34). A decreased L-arginine conversion to nitric oxide was detected after salt loading in the renal vascular endothelium of hypertensive patients (35). Finally, salt loading up-regulates the endogenous inhibitor of nitric oxide synthase, the asymmetrical dimethyl-L-arginine, which leads to decreased nitric oxide levels in mildly hypertensive patients (36).

There is a substantial body of evidence that aldosterone has multiple pathological effects on cardiac and vascular tissues but that they are closely linked to what has been referred to as "sodium status" (37). For instance, in rats maintained on a low-sodium diet (38) even high doses of aldosterone (plasma aldosterone levels 30 times greater than normal) do not cause vascular damage or a rise in blood pressure. This suggests that in normal circumstances a rise in plasma aldosterone is a physiological protective homeostatic response to compensate for the loss of sodium. The experiments described here are direct evidence that an inappropriate aldosterone/salt balance (i.e., medium to high aldosterone and raised plasma sodium) is crucial for endothelial stiffening and, possibly, for the development of hypertension. Such a view is similar to that in two editorial commentaries by Funder (39, 40). He coined the term "relative aldosterone excess" (39) consistent with the view that aldosterone would only turn into a harmful hormone when dietary salt intake was high.

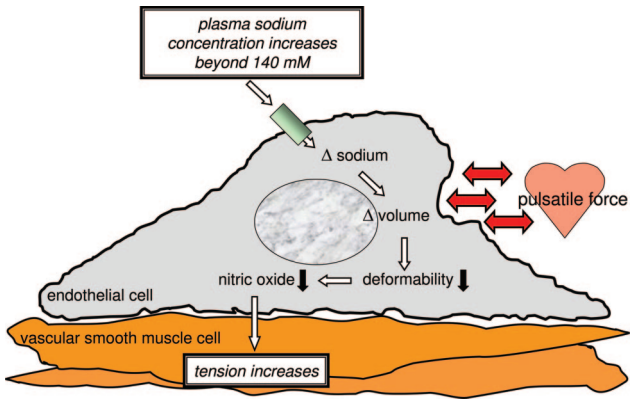

Fig. 7. Scheme defining the role of extracellular sodium in the regulation of vascular tone. Sodium enters the endothelial cell through ENaC. After a transient increase in cell volume, the endothelium stiffens. Thus vascular pulse pressure exerted by the working heart has a reduced impact on endothelial cell shape. The reduced rhythmic deformation of the endothelial cells decreases nitric oxide synthesis and release. As a result, vascular smooth muscle tone increases.

Fig. 7 shows a possible mechanism of the effect of sodium on endothelial cells. An acute rise in plasma sodium transiently alters cell volume. A prerequisite for this response is the pretreatment with aldosterone. By an as-yet-unknown mechanism, this causes an increase in endothelial stiffness and a partial loss of the cell's deformability which could result in a reduced release of nitric oxide (15). The lack of nitric oxide is supposed to increase vascular smooth muscle tone, which should increase total peripheral vascular resistance. In line with these deductions, the improvement of endothelial nitric oxide release by the β-blocker nebivolol does indeed increase arterial distensibility and thus lower blood pressure (41).

Many studies over the past 30 years report a positive correlation between dietary sodium intake and blood pressure. Clearly, nonacculturated populations who consume <3 g/day of NaCl have a very low incidence of hypertension in comparison to the Western industrial societies ingesting frequently >10g NaCl per day (reviewed in ref. 3). Careful measurements of plasma sodium in hypertensive individuals disclosed a small, but significant, increase in sodium concentration (17, 18). The present study suggests that such small changes in plasma sodium could have a large impact on endothelial function. Already a change of plasma sodium concentration of 5 mM, which still is considered to be in the physiological range, causes a change in endothelial stiffness by ≈10%. The data show a direct causative link between extracellular sodium and vascular endothelial morphodynamics. It is possible therefore that maintaining plasma sodium concentration and aldosterone concentrations in the low physiological range helps to keep arterial blood pressure normal and prevents vascular and end-organ damage (42).

Materials and Methods

Endothelial Cell Culture. Human endothelial cells [EA.hy926 (43)] were grown in culture as described (44). Chemicals were added to

the medium as appropriate. Aldosterone (d-aldosterone; Sigma–Aldrich, Steinheim, Germany) was dissolved in ethanol (1 mM stock solution, stored at 4°C for 2 weeks). Final concentration, measured in the culture medium (aldosterone ELISA; IBL, Hamburg, Germany) was 0.45 nM. Eplerenone (SC-66110; Pfizer, Kalamazoo, MI), a specific mineralocorticoid receptor antagonist, was dissolved in ethanol (2 mM stock solution, stored at −20°C). Final concentration in the experiments was 2 μM. We used the epithelial Na^+ channel blocker amiloride (Sigma–Aldrich), dissolved in water, at a final concentration of 1 μM. It should be noted that culture media maintained in regular dishes over 3 days in a standard incubator lose ≈10% of water. Therefore, culture dishes were placed close to a water surface to prevent evaporation.

Nitrite Measurements. Formation of nitric oxide was determined from the accumulation of nitrite (stable breakdown products of nitric oxide) in the culture medium (enriched by 250 μM L-arginine) of bovine aortic endothelial GM 7373 cells (DSMZ, Braunschweig, Germany). GM 7373 cells were used because basal nitric oxide metabolism is pronounced compared with EA.hy 926 cells and thus more suitable for nitrite analysis. In short, confluent GM 7373 cells (45) were cultured in T_{75} flasks in low sodium (135 mM sodium, in the absence or presence of 0.45 nM aldosterone) and high sodium (150 mM, in the absence or presence of aldosterone) medium. Mannitol (30 mM) was added to the low sodium medium for maintaining isoosmolality. FBS was reduced from 20% to 5% to prevent protein interference during nitrite analysis. Only 4.5 ml of medium was added to the individual culture flasks. Cultures were placed on a shaker inside the incubator and rhythmically shaken along the longitudinal flask axis (0.5 Hz). The culture medium was renewed every 24 h over 3 days. The harvested medium was centrifuged (134 × g), and the supernatant was stored at −20°C. The medium (pooled over 3 days) was pressed through a 30-kDa exclusion filter (Amicon Ultracell 30 K; Millipore, Schwallbach, Germany) by a 20-min centrifugation at 5,000 × g and 18°C. The filtrate (10 ml) was lyophilized, resuspended in 500 μl of H_2O, and again pressed through a filter (Amicon Microcon YM-3; exclusion size: 3 kDa) by a 3-h centrifugation at 14,000 × g and 18°C. Finally, the solution was mixed with Griess reagent (sulfanilamide and naphthalene-ethylenediamine dihydrochloride), and absorbance was measured spectrophotometrically at 546 nm. Nitrite concentration was determined by using a standard curve of $NaNO_2$ (1–100 μM). Stepwise filtering was necessary to remove all proteins from the solution. Lyophilization and subsequent resuspension in a small volume (concentration factor = 20) shifted nitrite concentration to a better detection range. Rhythmic shaking of the culture flasks mimicked shear stress to raise nitric oxide formation.

Endothelial Cell Stiffness Measurements. The stiffness of living EA.hy 926 endothelial cells was measured with soft cantilevers (MLCT-contact microlevers, spring constant: 0.01 N/m; Veeco, Mannheim, Germany). The AFM tip was pressed against the cell so that the membrane was indented (Fig. 1), which distorts the AFM cantilever, which serves as a soft spring. The cantilever deflection, measured by a laser beam when reflected from the gold-coated cantilever, permits force-distance curves of single cells. The slope of such curves is directly related to the force (expressed in newtons), defined here as stiffness, necessary to indent the cell (indentation depth: 200 nm). Measurements were made on single cells in paired fashion, i.e., sodium was increased stepwise and stiffness was measured after an equilibration period of a few minutes. Experiments were performed on living cells at 37°C by using a Multimode AFM (Veeco) and a feedback-controlled heating device. The cells were bathed in Hepes-buffered solution (140 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 10 mM Hepes, pH 7.4).

Statistics. Data are shown as mean value ± SEM. Significance of differences was evaluated by the paired or unpaired Student's t test if applicable. Overall significance level is $P = 0.05$.

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