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Endothelial cells as vascular salt sensors

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Dietary sodium and potassium contribute to the control of the blood pressure. Endothelial cells are targets for aldosterone, which activates the apically located epithelial sodium channels. The activity of these channels is negatively correlated with the release of nitric oxide (NO) and determines endothelial function. A mediating factor between channel activity and NO release is the mechanical stiffness of the cell's plasma membrane, including the submembranous actin network (the cell's 'shell'). Changes in plasma sodium and potassium, within the physiological range, regulate the viscosity of this shell and thus control the shear-stress-dependent activity of the endothelial NO synthase located in the shell's 'pockets' (caveolae). High plasma sodium gelates the shell of the endothelial cell, whereas the shell is fluidized by high potassium. Accordingly, this concept envisages that communications between extracellular ions and intracellular enzymes occur at the plasma membrane barrier, whereas 90% of the total cell mass remains uninvolved in these changes. Endothelial cells are highly sensitive to extracellular sodium and potassium. This sensitivity may serve as a physiological feedback mechanism to regulate local blood flow. It may also have pathophysiological relevance when sodium/potassium homeostasis is disturbed.

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POSSIBLE CLINICAL RELEVANCE OF ENDOTHELIAL CELL STIFFNESS

In physics, the term *stiffness* is clearly defined: 'Stiffness is a measure of the resistance offered by an elastic body to deformation'. In cellular physiology stiffness is the force (Newton) necessary to compress a cell for a certain distance (meter). Force is applied to most tissues in real life, particularly to vascular endothelium. Hemodynamic forces, generated by the beating heart, give rise to shear stress at the endothelial surface. It is inevitable therefore that the apical cell surfaces undergo reversible deformations and that it is this mechanical stimulus that triggers the activity of the endothelial nitric oxide synthase (eNOS) and the release of nitric oxide (NO).[1] The NO diffuses to the adjacent vascular smooth muscle cells, which relax leading to vasodilation. This regulatory mechanism distributes the blood in the organism according to the metabolic demands whereas the systemic blood pressure is maintained within physiological limits. The same shear force should cause a stiff (less deformable) cell to release less NO. Therefore, endothelial mechanical stiffness is a key parameter in the control of local blood supply and arterial blood pressure (Figure 1).

ATOMIC FORCE MICROSCOPE: A MECHANICAL NANOSENSOR

The 'tool of choice' for quantitatively measuring the stiffness (given in N/m) of living adherent endothelial cells is an atomic force microscope (AFM). In principle, the AFM is used as a mechanical tool. The tip of the AFM (sometimes shaped to be a small sphere) is pressed against the cell so that the membrane is indented (Figure 2). This distorts the cantilever on the AFM that serves as a soft spring. The cantilever deflection, measured by a laser beam, is reflected from the gold-coated cantilever surface, which permits force–distance curves of single cells to be measured. The slope of such curves is directly related to the force (expressed in Newton) necessary to indent the cell for a given distance (expressed in meter). At least two different slopes can be identified depending on the depth of indentation. The initial rather flat slope (indentation depth: up to a several 100 nm) reflects the stiffness of the soft plasma membrane including the cortical cytoskeleton (the cell's 'shell'), whereas the late rather steep slope reflects the stiffness of the more rigid cell center.[2]

SODIUM: 'STIFFENER' OF VASCULAR ENDOTHELIAL CELLS

For more than three million years primitive man consumed less than 1 g per day of sodium chloride.[3] About 8000 years ago, with the advent of agriculture and farming,

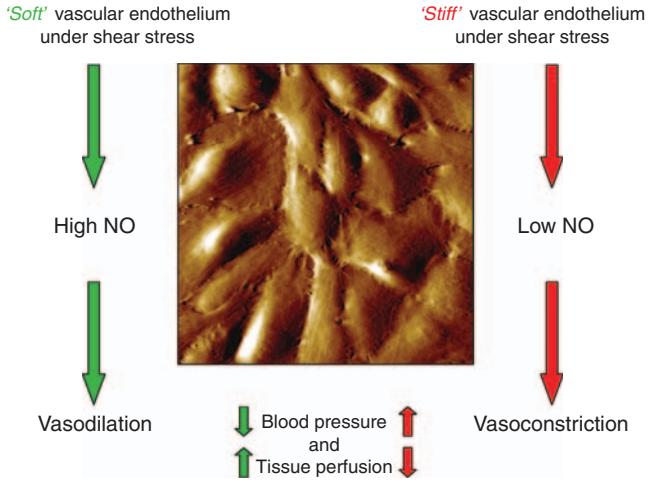

Figure 1 | Concept of how the mechanical stiffness of endothelial cells participates in the regulation of blood pressure.

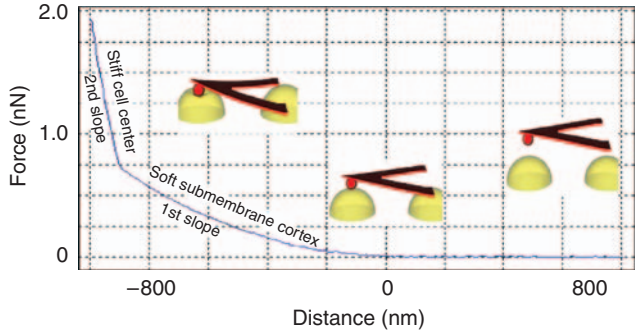

Figure 2 | Indentation technique using atomic force microscopy. Indentation curve with two different slopes (modified from Oberleithner *et al.*[15]).

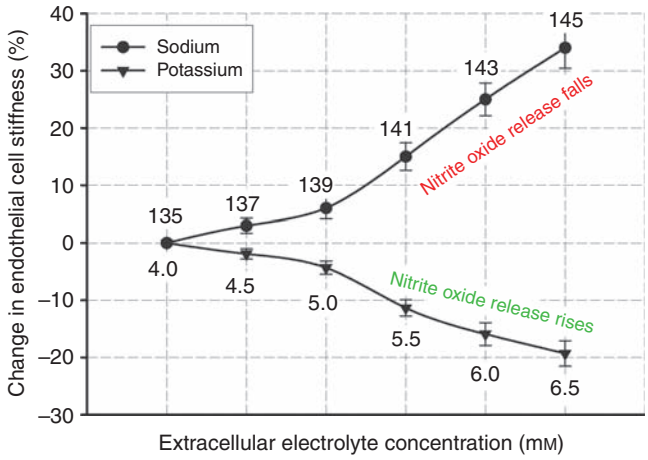

Figure 3 | Relationship between cell stiffness and electrolyte concentrations. Please note that the electrolyte-induced changes in stiffness occur within minutes. Numbers close to the mean values (± s.e.m.) are the respective ion concentrations in the extracellular solution (mmol/l) (modified from Oberleithner *et al.*[9,15]).

sodium chloride consumption increased to about 10 g per day. The main reason was to preserve foods such as bread and meat. Salt became precious because it allowed food to be stored for prolonged periods.[4] For instance Christopher Columbus would have never discovered the West Indies without the use of salt to keep fish, meat, and bread from perishing. Although the German engineer Carl von Linde invented the refrigerator more than 130 years ago, which has enabled food to be stored by deep-freezing, modern society has not yet returned to the low levels of sodium consumed during primitive times. As life expectation is nowadays close to 80 years, the harmful effects of high-salt diets has become increasingly apparent. Hypertension, stroke, coronary heart disease, and renal fibrosis are related to a high sodium intake.[5] Although the deleterious effects of a high sodium intake are now obvious, the underlying mechanisms how sodium chloride exerts its effect at the organ, tissue, and cellular levels are still unclear. A high sodium intake causes fibrosis and inflammatory processes in the kidney and heart.[6] When dietary salt intake exceeds renal excretory capacity an increased amount of sodium is stored in the space between cells, bound to extracellular organic material.[7] Plasma sodium is raised in hypertension (by 3–4 mmol/l) when dietary sodium intake is raised.[8] It has therefore been postulated that changes in plasma sodium *per se* may control the blood pressure. Accordingly, the effect of such small changes in sodium concentration on endothelial function has been studied. It was found that when extracellular sodium is raised in this manner endothelial cells stiffen within minutes[9] and that this only occurs in the presence of aldosterone. It is remarkable, however, that endothelial cells appear insensitive to sodium concentrations below 139 mmol/l, above which, however, they are highly sensitive (Figure 3). This type of experiment is technically challenging as buffered solutions that differ by only 2 mmol/l sodium are difficult to prepare and handle. It is emphasized that the endothelium's response to the changes in sodium concentration is strictly dependent on aldosterone. Inhibition of the cytosolic mineralocorticoid receptors by spironolactone (or eplerenone) prevents endothelial stiffening as does inhibition of the epithelial sodium channel (ENaC; present in the apical plasma membrane of endothelia) by amiloride.[9] These *in vitro* experiments may explain the protective action of these two substances on the cardiovascular system.[10,11] The data strongly support the view that the blood vessels and the heart are primary targets for diuretics, independent of any actions they may have on the kidney.

POTASSIUM: SOFTENER OF VASCULAR ENDOTHELIAL CELLS

Besides a genetic predisposition for hypertension,[12] it is the amount of the daily sodium and potassium intake that may lead to onset of elevated blood pressure in the otherwise healthy individual. In contrast to natural food, processed food products are rich in sodium and poor in potassium. There is general agreement that a high-potassium, low-sodium diet exerts beneficial effects on the cardiovascular system[13] and may even influence emotion such as

depression, tension, and vigor.[14] Potassium deficiency is difficult to detect for 98% of body potassium is intracellular. Plasma potassium is maintained within narrow limits and is a subtle indicator for any disturbances of potassium homeostasis. For this reason we studied the effect of extracellular potassium on endothelial cells (Figure 3). It was found that extracellular potassium concentrations above 5 mmol/l swell and soften endothelial cells.[15] Plasma potassium is often raised in kidney disease but 'local' potassium concentrations greater than 5 mmol/l, are absolutely normal in muscle during physical exercise[16] and in the brain during increased neuronal activity[17] while overall electrolyte homeostasis is unaffected.

The extent of potassium-induced cell softening depends on the absence or presence of aldosterone and the concentration of extracellular sodium. When extracellular sodium is low and aldosterone is absent (an experimental rather than a physiological condition), a 27% decrease in endothelial cell stiffness can be induced by raising the concentration of potassium from 4 to 6 mmol/l. In the presence of aldosterone and extracellular sodium in the low physiological range (a more realistic situation in humans), the same maneuver causes stiffness to decrease by 21%. However, if aldosterone is present and extracellular sodium is in the high range (a pathophysiological condition), then the decrease in stiffness induced by a rise in potassium from 4 to 6 mmol/l is only about 6%, a change which is not statistically significant.[15] It is true that these observed interactions between extracellular sodium and potassium are taking place under well-controlled conditions *in vitro*. Though the situation may be different *in vivo*, it is clear that stiffness and NO release are inversely related.

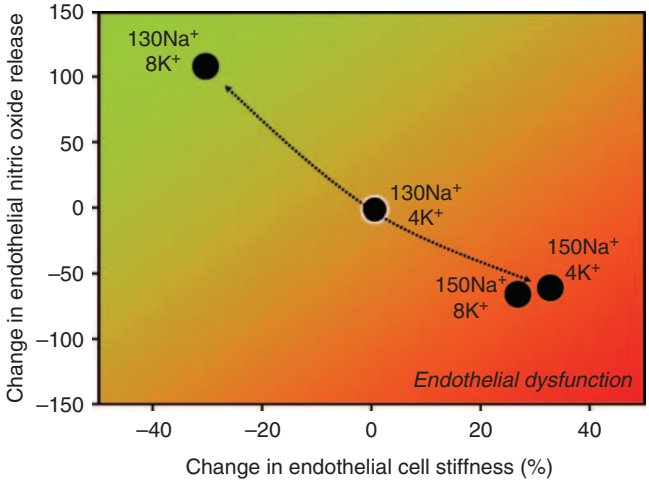

Figure 4 | Negative correlation between cell stiffness and nitric oxide (NO) release. NO release was derived from the nitrite concentrations measured in the supernatant culture media. Data were taken from Oberleithner *et al.*[9,15]

ENDOTHELIAL NO RELEASE: OPPOSING ROLES OF SODIUM AND POTASSIUM

Endothelial nitric oxide synthase is located at the caveoli of the apical cell membrane and its expression and/or activity is regulated by various factors. It is stimulated by increases in intracellular Ca^{2+} (through calmodulin) independent of the initial stimulus. Besides the fact that NO synthase isoenzymes are associated with more than 20 interacting proteins,[18] it is likely that sodium ions control eNOS activity, which is also inhibited by aldosterone,[19] possibly indirectly through EnaC-mediated sodium influx. Conversely, inhibition of ENaC-mediated sodium influx by amiloride activates eNOS.[20] An increase in the intake of salt induces the production of asymmetrical dimethyl-L-arginine, which is a competitive eNOS inhibitor,[21] and increasing extracellular sodium within the physiological range, as illustrated in Figure 2, downregulates eNOS expression and angiogenesis.[22] In Figure 4 data from two reports[9,15] on cell stiffness and NO release (measured as nitrite in the supernatant of shear-stress-activated endothelial cells *in vitro*) are combined in a single graph. It is noticeable that (1) there is a negative correlation between stiffness and eNOS activity, (2) extracellular sodium concentration strongly determines stiffness and eNOS function, and (3) extracellular potassium concentration influences eNOS activity and stiffness only at low sodium concentrations. All the experiments contributing to Figure 4 were performed in the presence of concentrations of aldosterone similar to those found in physiological conditions.

A big question remains: does eNOS activity depend on cell stiffness (as proposed above) or *vice versa*? In other words does the cell's deformability determine eNOS activity or does this activity 'itself' modify the cell's mechanical compliance to enable the endothelium to better adjust to the large changes in morphology that inevitably occur when blood vessels recurrently dilate and contract?

'SOLATION–GELATION' HYPOTHESIS

Vascular endothelial cells undergo large changes in shape (e.g., during vascular dilation/constriction, particularly in those that occur with each contraction of the heart) and can best adjust to such alterations if the deformability (physical compliance) of the cells is high. The AFM analysis reveals at least two linear slopes in the indentation curves; the first tends to be flat whereas the second is steeper (Figure 2). The first flatter slope indicates a low stiffness, which is limited to the submembranous cortex of the cell (the cell's shell). The nature of the slope indicates clearly that there is a fluidic layer immediately beneath the plasma membrane, which is highly changeable in terms of thickness and viscosity. The cortical cytoskeleton of vascular endothelial cells is highly 'dynamic' and the state of polymerization of cortical actin determines the structure and mechanical properties of this layer.[2,23] Monomeric globular actin (G-actin), which can rapidly polymerize into filamentous actin (F-actin), can cause a rapid increase in local viscosity (gelation). Alternatively, switching from F-actin to G-actin using the polymerization inhibitor cytochalasin *D* is associated with solation of the cortex (i.e., the submembranous multimeric actin filament meshwork

disaggregates into actin monomers).[15] An increase in extracellular potassium mimicks this response indicating that potassium *per se* (or, more indirectly, through changes of the membrane potential; see below) softens the cortical actin cytoskeleton by changing F-actin to G-actin. G-actin is known to colocalize with eNOS and to increase eNOS activity.[24] This could explain the activation of eNOS by high potassium.

It is possible that in this system sodium is a functional antagonist. Sodium influx, mediated by aldosterone-activated ENaC, stiffens the cytoskeleton by increasing the viscosity of the submembranous layer. It is hypothesized that when sodium is in the high physiological range, filamentous actin dominates over monomeric actin. This would explain the sodium-induced increase in cell stiffness. When potassium is elevated, actin filaments disaggregate into actin monomers and endothelial cells soften. Both F-actin and G-actin are negatively charged molecules and their interaction with Na^+ and K^+ will finally depend on the local concentrations and specific affinities of the respective ions. It has to be kept in mind that theses changes are supposed to take place in a relatively restricted cytosolic space, directly beneath the plasma membrane, most likely at the caveolae.[1] As this cytosolic submembranous zone (cell shell) is only a few hundred nanometers thick, it is implied that about 90% of the cell's body remains uninvolved.

The solation–gelation hypothesis, based on the different interaction between sodium and potassium with the submembranous actin network, is supported by the observation that sodium has a greater affinity to protein surfaces than potassium.[25] It is assumed that when sodium enters the cell (e.g., through ENaC activation) it binds with high affinity to actin displacing potassium from the carboxylate groups within the amino-acid side chains. Thus, increasing the concentration of sodium, which has a higher affinity to actin as compared to potassium, effectively modulates its protein–protein interaction strength.[25] Such small changes in sodium or potassium in the submembranous zone should control the state of actin polymerization and thus the cell stiffness and functionality (Figure 5).

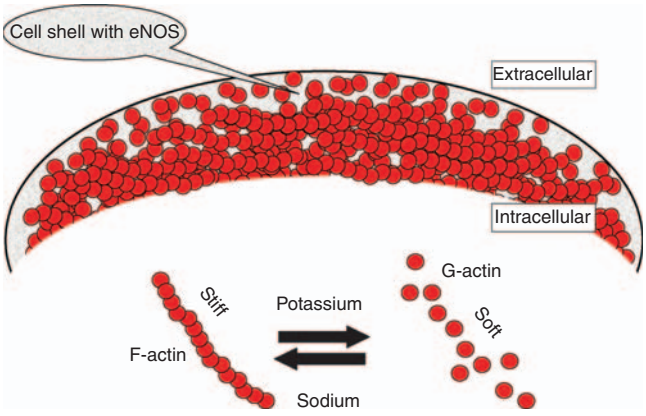

Figure 5 | Hypothesis for how sodium and potassium control the fluidity of the cortical zone ('cell shell') in an endothelial cell.

'ELECTRIC FIELD' HYPOTHESIS

The membrane potential of endothelial cells is highly variable.[26] One factor that may cause this variability is a change in potassium flux across the cell membrane. At low extracellular potassium concentrations, an increase can, paradoxically, hyperpolarize the membrane potential of endothelial cells.[27] Such a change in membrane potential may directly or indirectly affect the state of polymerization of the submembranous actin network and thus modify the cell's stiffness.[28]

There is a link between the plasma membrane potential and NO synthesis. In human vascular endothelial cells, calcium-activated potassium channels directly control NO synthesis.[29] Activation of these channels hyperpolarizes the endothelial cell leading to increased eNOS activity. In this functional state the cell is soft, that is, beneath the plasma membrane G-actin dominates over F-actin.

In contrast, active ENaCs depolarize the endothelial cell. In this functional state the cell is stiff, that is, F-actin dominates over G-actin. Cell stiffening can be prevented by amiloride, a diuretic that blocks ENaC and hyperpolarizes the endothelial cell, or by spironolactone, an aldosterone receptor blocker that prevents ENaC from being expressed in the endothelium.[30]

CLINICAL PERSPECTIVES

The finding that endothelial cells modify their function in response to changes in the extracellular concentration of sodium and potassium (Figure 6) could trigger new approaches in the diagnosis and therapy of cardiovascular disease. The control of local tissue perfusion strongly depends on the local environment that includes electrolyte concentrations that differ depending on the metabolic activity of the respective site or organ. It seems promising to test ion channel blockers and agonists, well known to exert their effects on various epithelia, including vascular endothelium. It may turn out that some of the actions of such substances are due to alterations of vascular function rather than to modifications of epithelial transport.

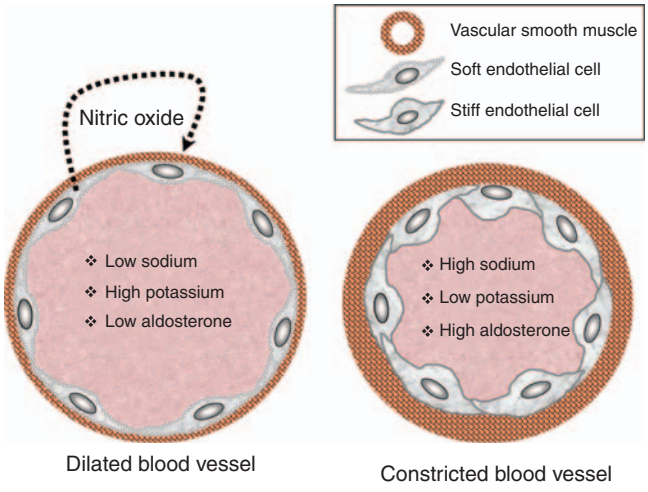

Figure 6 | Concept of how sodium, potassium, and aldosterone contribute to the regulation of blood vessel tone.

DISCLOSURE
All the authors declared no competing interests.

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