Sodium Channels

Endothelial Sodium Channels Trigger Endothelial Salt Sensitivity With Aging

Moritz Paar, Hermann Pavenstädt, Kristina Kusche-Vihrog, Verena Drüppel, Hans Oberleithner, Katrin Kliche

See Editorial Commentary, pp 231–232

Abstract—The epithelial sodium channel is also expressed in vascular endothelium (endothelial sodium channel \(\text{[EnNaC]}\)). Depending on ambient sodium concentration, EnNaC is associated with mechanical stiffening of the endothelial cell cortex, leading to endothelial dysfunction. Because the incidence of both salt sensitivity and endothelial dysfunction increases with age, we investigated the abundance of EnNaC in aging mice. To assess EnNaC functionality and endothelial salt sensitivity, stiffness was measured while ambient sodium was varied. Aortae of young (3 months) and old (15 months) C57BL/6J wild-type mice were kept ex vivo on a physiological concentration of aldosterone (0.45 mmol/L). Spironolactone (10 nmol/L) and amiloride (1 μmol/L) were applied for aldosterone antagonism and EnNaC blockage, respectively. EnNaC at the endothelial cell surface was quantified by immunofluorescence staining. Cortical stiffness was monitored by atomic force microscopy when ambient sodium was raised from 135 to 150 mmol/L. In ex vivo aortae of older mice, endothelial cells had significantly higher EnNaC numbers than those of younger mice (+23%). In parallel, cortical stiffness was found increased (+8.5%). Acute application of high sodium led to an immediate rise in stiffness in both groups but was pronounced in endothelium of older mice (+18% versus +26%). Spironolactone and amiloride lowered EnNaC abundance and prevented endothelial stiffening under all conditions. We conclude that EnNaC mediates endothelial salt sensitivity in the aging process. This mechanism might contribute to the development of age-related cardiovascular disease and suggests the usage of spironolactone and amiloride specifically in the elderly. (Hypertension. 2014;64:391-396.) ● Online Data Supplement

Key Words: aging ■ endothelium, vascular ■ sodium channels

The role of salt in the development of hypertension is subject of diverse studies and discussions. In this regard, salt sensitivity is usually defined by a significant decrease in mean arterial blood pressure when dietary salt intake is lowered.1 Following this definition, it is estimated that at least one third of the world’s population is sensitive to sodium.2 These subjects have higher levels of serum markers of inflammation and of vascular remodeling, possibly explaining the greater risk of cardiovascular disease that is present in these individuals.3 Moreover, salt sensitivity is associated with more severe target organ injury and higher mortality once cardiovascular disease is manifest. Notably, this is the case even in normotensive subjects.4 Although evidence identifies salt as a crucial mediator of cardiovascular disease, little is known about the underlying mechanisms on a cellular level. High sodium concentrations have been shown to stiffen vascular endothelial cells (ECs) mechanically, accompanied by a decrease in the bioavailability of nitric oxide.5 Because the release of this vasodilatory, antiatherogenic gas is mainly stimulated by shear stress,6 it is the mechanical alteration itself that is thought to cause the dysfunction of the EC.7 In the process of sodium-induced EC stiffening and EC dysfunction, the presence of sodium channels in vascular endothelium seems to be of particular importance.8 The epithelial sodium channel is also expressed in vascular endothelium and has been termed endothelial sodium channel (EnNaC).9 As in epithelia, synthesis and activation of EnNaC is regulated by the mineralocorticoid hormone aldosterone.10 Specific EnNaC blockade completely prevents sodium-induced stiffening.5 This strongly suggests EnNaC as the crucial mediator of endothelial salt sensitivity. Considering that EC dysfunction is followed by hypertension, wall thickening, vascular stiffening, and atherosclerosis, EnNaC and endothelial salt sensitivity might play an important role in linking the pathophysiology of salt and cardiovascular disease.

Another independent risk factor for cardiovascular disease is age.11 Correspondingly, endothelial function has been...
shown to decline with age.\textsuperscript{12} Interestingly, the process of aging is associated with enhanced salt sensitivity in hypertensive humans.\textsuperscript{13} The detailed mechanisms of increasing salt sensitivity in the elderly are currently unknown but have, in part, been attributed to an age-dependent decline in the sodium excretion ability of the kidneys.\textsuperscript{14}

Based on the above-mentioned findings, we assumed a link between systemic salt sensitivity and endothelial salt sensitivity at age. We, therefore, investigated EnNaC abundance in ex vivo aortae of young (3 months) and old (15 months) mice. To test EnNaC functionality and endothelial salt sensitivity, the stiffness of the EC cortex was measured in living ex vivo aortae while ambient sodium was varied. Endothelial salt sensitivity was defined as a significant rise in stiffness because of an acute increase in ambient sodium from 135 mmol/L to 150 mmol/L. Spirotonolactone and amiloride were applied for aldosterone antagonism and specific EnNaC blockade, respectively.

Methods

Removal and Culture of Ex Vivo Aortae

All procedures were conducted in accordance with the declaration of Helsinki and were approved by the local committee for animal care. C57BL/J6 male wild-type mice were purchased from Charles River (Charles River laboratories, France) at 3 months (young) and 15 months (second half of life, old) of age. Animals had free access to maintenance diet (0.2% sodium; Altromin, Lage, Germany) and water. Mice were killed and aortae were removed as recently described.\textsuperscript{15} Small vessel patches \(\approx 1 \text{ mm}^2\) in size were attached on Cell-Tak (BD Bioscience)-coated glass cover slips with the endothelial surface facing upward. Aortic preparations were then incubated at 37°C, 5% CO\(_2\), and 100% humidity for 48 hours in Gibco minimal essential medium (Invitrogen, Karlsruhe, Germany) supplemented with 20% fetal bovine serum (PAA Clone, Coelbe, Germany) and with 1% penicillin G, streptomycin, minimal essential medium vitamins, and minimal essential medium nonessential amino acids (all purchased from Invitrogen). Sodium concentration of the final medium was 135 to 137 mmol/L. All vessel patches from 1 individual aorta were randomly split into 3 different groups. To mimic in vivo conditions, aldosterone (Sigma-Aldrich, Steinheim, Germany) was added in a final concentration of 0.45 nmol/L (physiological range) to the medium of all groups. For mineralocorticoid receptor (MR) antagonism, spironolactone (Sigma-Aldrich) was added in a final concentration of 10 nmol/L. For EnNaC blockade, amiloride (Sigma-Aldrich) was added in a final concentration of 1 μmol/L. Before the actual experiments, integrity of the EC monolayer after the process of preparation and cultivation was repetitively tested by vascular endothelial cadherin immunostaining. Please see the online-only Data Supplement for details.

Quantification of EnNaC Molecules in the EC Surface

Immunofluorescence staining of single α-EnNaC molecules in the EC surface using quantum dots (QD) and acquisition of immunofluorescence images were performed as described.\textsuperscript{16} Aortic preparations were fixed in glutaraldehyde (final concentration, 0.1%) for 30 minutes. Because the α-EnNaC subunit is crucial for EnNaC function, a primary polyclonal rabbit anti–α-epithelial sodium channel antibody (Santa Cruz, Heidelberg, Germany; dilution 1:250) was applied. Secondary antibodies were QD labeled (Qdot 655 goat anti-rabbit IgG, Invitrogen, Eugene, OR; dilution 1:800). Immunofluorescence images were acquired with an inverted fluorescence microscope (Axiovert 200, Zeiss, Oberkochen, Germany) equipped with a 63×1.45 water immersion objective using the QD 655 filter (420 nm excitation, 655 emission; XF302-1 filter, Omega Optical, Brattleboro, VT). To facilitate the location of the EC level, cell nuclei were stained by adding 4′,6-diamidino-2-phenylindole (Invitrogen; 0.0013%) to the fluorescent mounting medium (DAKO, Carpinteria, CA). A representative image is given in Figure S1B in the online-only Data Supplement. Images were taken on 3 different focal planes at a distance of 0.5 μm and were superimposed for QD analysis. The number of QD per 1000 μm\(^2\) image area was counted manually and corrected for QD background levels in negative controls (data not shown). Results are given in QD per 1000 μm\(^2\) EC surface area (N=number of analyzed aortae, n=number of analyzed regions).

Measurements of EC Stiffness

Stiffness of the EC cortex was assessed by atomic force microscopy on living ex vivo aortae as described by Kusche-Vihrog et al\textsuperscript{2} and Drieppe et al.\textsuperscript{10} During atomic force microscopy experiments, aortic preparations were continuously perfused with HEPES-buffered solution (in mmol/L: 5 KCl, 1 MgCl\(_2\), 1 CaCl\(_2\), 5 glucose and 10 HEPES) containing the agent(s) of the respective treated group. At the beginning of each experiment, HEPES buffer containing 135 mmol/L sodium (normal) was used, and cortical stiffness of 20 individual ECs was assessed. Next, the buffer was replaced by HEPES buffer containing 150 mmol/L sodium (high), and for the following 15 minutes, the stiffness of 1 single cell was monitored continuously (paired experiment). Artefacts resulting from continuous indentation were excluded by control experiments under persistent normal sodium conditions. These data are shown in Figure S2. Finally, cortical stiffness of another 20 ECs was measured (unpaired experiment). Osmolarity of the normal sodium HEPES buffer was adjusted to the osmolarity of high sodium by the addition of mannitol (N=number of analyzed aortae, n=number of analyzed cells).

Blood Pressure Measurements

Blood pressure measurements were performed with the CODA non-invasive blood pressure system (Kent Scientific, Torrington, CT) with a tail cuff as described by Feng and DiPetrillo.\textsuperscript{17} For detailed information, please see the online-only Data Supplement (N=number of animals, n=number of measurements).

Statistical Analysis

OriginPro 8 (OriginLab Corporation, Northampton, USA) was used for statistical exploration of unpaired data. Parametric data are expressed as mean±SEM and were tested for significance with the unpaired Student\textsuperscript{t} test. Nonparametric data are expressed as medians (first quartile [Q1], third quartile [Q3]) and were tested for significance with the Mann–Whitney U test. Paired data were analyzed with repeated measures ANOVA and Scheffe post hoc test using StatSoft STATISTICA version 9.1 (StatSoft, Inc, Tulsa, OK). The significance level is represented as *P<0.05 or #P<0.05.

To present unprocessed results, data are displayed in boxplots if applicable. Here, each individual value is represented by 1 single dot. In addition, the boxplots contain the following descriptive statistics: median (horizontal line), mean (filled square), interquartile range (box), and outlier (whiskers).

Results

EnNaC Abundance in Ex Vivo Aortae of Young and Old Mice

Examples of immunofluorescence images are shown in Figure S3. As presented in Figure 1, EC in ex vivo aortae of young mice showed 12.0 QD/1000 μm\(^2\) EC surface area (Q1: 9.0, Q3: 15.0). The quantification in older animals revealed 14.8 QD/1000 μm\(^2\) EC surface area (Q1: 12.2, Q3: 17.8). Thus, the amount of EnNaC was significantly higher in EC in ex vivo aortae of older animals (+23%).

In the presence of the MR antagonist spironolactone, the number of QD was 6.0/1000 μm\(^2\) EC surface area (Q1: 4.4, Q3: 7.9) in young animals and 7.7/1000 μm\(^2\) EC surface area (Q1: 5.8, Q3: 9.7) in older ones. In comparison with the untreated control of the respective age group, this represents...
a reduction of EnNaC channels by ≈50% as a result of spironolactone treatment. When the specific EnNaC blocker amiloride was added to the medium, EC of young mice contained 3.2 QD/1000 μm² EC surface area (Q1: 2.0, Q3: 5.4). In older ones, 6.0 QD/1000 μm² EC surface area (Q1: 4.7, Q3: 8.7) could be found. Therefore, amiloride reduced the number of EnNaC in older mice by 59% and in young ones by 73.3%.

EC Stiffness in Ex Vivo Aortae of Young and Old Mice

Figure 2 shows that under normal sodium concentration, the stiffness of EC in ex vivo aortae of young mice was 1.17±0.006 pN/nm. The ECs of older mice had a cortical stiffness of 1.27±0.008 pN/nm and were thus significantly stiffer (+8.5%).

In the presence of spironolactone, the EC stiffness of young animals was 0.98±0.008 pN/nm and in older ones 1.09±0.009 pN/Nm. In presence of amiloride, EC in young mice had a stiffness of 0.98±0.01 pN/nm and in older ones of 1.05±0.009 pN/nm. Hence, treatment of the ex vivo aortae with spironolactone and amiloride significantly reduced endothelial stiffness in both age groups.

Endothelial Salt Sensitivity in Ex Vivo Aortae of Young and Old Mice

To assess salt sensitivity of ECs under the different experimental conditions, cortical stiffness was analyzed when ambient sodium concentration was raised from 135 mmol/L to 150 mmol/L. During the first 15 minutes after this rise, development of cortical stiffness in the control group was continuously monitored (Figure 3). A rapid increase in cortical stiffness could be observed in both age groups that was virtually parallel (minute 5 in young and minute 6 in older endothelium). Then, the stiffness of EC in young mice remained constant (steady state at minute 11), whereas EC in older animals continuously stiffened until minute 15.

Next, the cortical stiffness was measured in presence of the high ambient sodium concentration when the steady state was reached in all groups (Figure 4). Under control conditions, ECs of young mice showed a stiffness of 1.38±0.006 pN/nm. This is equivalent to a rise of 18% because of high sodium. ECs of older mice became remarkably stiffer (1.60±0.01 pN/nm). This corresponds to an increment of 26% because of high sodium. Consequently, ECs of older animals were more sensitive to salt than young ones. Spironolactone and amiloride significantly reduced endothelial salt sensitivity in both age groups (spironolactone young: 1.07±0.007 pN/nm, old: 1.16±0.009 pN/nm; amiloride young: 1.03±0.009 pN/nm, old: 1.13±0.009 pN/nm).

Blood Pressure in Young and Old Mice

As the Table displays, systolic arterial blood pressure of young mice was 105.9±1.12 mm Hg. Older mice exhibited a significantly higher systolic arterial blood pressure (111.4±1.14 mm Hg). Also diastolic arterial blood pressure was significantly higher in older mice than in the young ones (84.7±1.23 versus 77.2±1.00 mm Hg). Consequently, mean arterial blood pressure became higher in the older mice than in the young ones (190.5±3.81 mm Hg versus 183.1±2.59 mm Hg).
Discussion

Aging is a major risk factor for cardiovascular disease, but the underlying mechanisms are not yet fully understood. The basic concept of most aging theories is that damage accumulates over time, leading to age-dependent molecular, cellular, and organ changes. Furthermore, a genetically predetermined aging program is discussed.

In the present study, we found that EnNaC abundance increases with age. Older endothelia were significantly stiffer and exhibited higher increments in stiffness because of acute high sodium. Per definition, this implicates an increase in endothelial salt sensitivity in the process of aging. The fact that the specific EnNaC blocker amiloride prevented salt-induced stiffening identifies EnNaC as the mediator of aging-induced endothelial salt sensitivity. Taking the crucial role of EC function in the pathophysiology of the cardiovascular system into account, our results represent a cellular mechanism how systemic salt-sensitive hypertension and cardiovascular disease may develop in the elderly (Figure 5). The detection of elevated blood pressure in the older animals further highlights the physiological relevance of the present results. The causal relationship of elevated EnNaC and elevated blood pressure, of course, cannot be deduced from the actual experiments. Other mechanisms certainly contribute to the development of hypertension with age. It is even conceivable that the high blood pressure causes the elevation in EnNaC expression and not vice versa. However, nothing is known about such potential phenomena.

The question arises how EnNaC abundance increases with age. It was shown that the MR is increased in whole vessel preparations of old rats and in an in vitro model of aging endothelia. In the latter, EnNaC mRNA was found elevated, too. Considering that aldosterone was present in all experimental groups, the increase in EnNaC might, therefore, be the result of increased agonist/receptor interaction followed by gene transcription. This view is supported by the observation that spironolactone was able to attenuate the aging-related increase in EnNaC. However, spironolactone-induced EnNaC suppression in older ECs did not reach the same level as in younger ones. Because spironolactone is a competitive antagonist of the MR, this might be a result of the increased agonist/receptor interaction while the dosage of spironolactone was consistently low. Moreover, MR-independent mechanisms in the age-associated EnNaC increase are conceivable. In this case, as for the mechanism of MR increase, we suggest that aging-related changes in gene expression and in protein function might be the cause.

The link between EnNaC and mechanical stiffness was demonstrated before. Because the mechanical stiffness of the EC cortex is mainly determined by the F-actin over G-actin ratio, the EnNaC protein itself or the EnNaC-mediated sodium influx are obviously able to influence the polymerization state of the cortical actin web. The fact that (1) EnNaC-mediated sodium influx is able to significantly increase intracellular sodium concentration and that (2) stiffness increased almost immediately after exposure to high sodium points toward a crucial role of sodium influx in this mechanism. Interestingly, Na+/K+ ATPase is known to transport out of the EC might contribute to the prolonged

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Young</th>
<th>Old</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>SAP, mmHg</td>
<td>105.9±1.1</td>
<td>111.4±1.1</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>DAP, mmHg</td>
<td>77.2±1</td>
<td>84.7±1.2</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>MAP, mmHg</td>
<td>86.5±1</td>
<td>92.7±1</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

SAP, DAP, and MAP of young and older mice (N=4, n=134–142). DAP indicates diastolic arterial blood pressure; MAP, mean arterial blood pressure; and SAP, systolic arterial blood pressure.
endothelial stiffening attributable to high sodium observed in the older age group.

Dermal epithelium and cardiac myocytes have been shown to stiffen with age, too.\textsuperscript{28,29} Nothing is known about ENaC abundance in these types of cells, and hence authors discuss altered cytoskeletal gene expression as a reason for age-related stiffening. Because actin expression in ECs has also been found altered at age,\textsuperscript{30} this might contribute to the aging-related EC stiffening, too. In concordance, amiloride and spironolactone attenuated aging-induced stiffening in the present experiments but did not completely prevent it.

Given that aldosterone was present in all our experimental groups, the role of aldosterone in endothelial salt sensitivity needs to be discussed. It is well known that aldosterone has adverse effects on the cardiovascular system that are independent of blood pressure. Concomitantly, aldosterone has been shown to participate in vascular remodeling by directly acting on different components of the blood vessel.\textsuperscript{31} However, in vivo data were often gained in animal models that did not only receive aldosterone but that were also on a high-salt diet.\textsuperscript{32} High aldosterone had no effect on rats when they received low salt,\textsuperscript{33} and the inflammatory response to MR activation only occurred in the presence of sodium excess.\textsuperscript{34} Therefore, effects formerly attributed to aldosterone are now rather attributed to the salt itself.\textsuperscript{35} A closer look on our experiments reveals that in the virtual absence of aldosterone action, as indicated in the spironolactone group, the acute rise in sodium failed to influence EC stiffness markedly. Only in the presence of aldosterone and in the absence of spironolactone (ie, in the control group) sodium was effective. We conclude that aldosterone (via ENaC) is required to render vascular endothelium sensitive to sodium. Aging-induced enhanced EC sensitivity to aldosterone/salt might contribute to the lower systemic levels of renin and aldosterone\textsuperscript{36} and to the increased systemic salt sensitivity in spite of unchanged or even decreased plasma sodium concentrations\textsuperscript{37,38} that are observed at age.

Considering that aldosterone is usually present in the organism, our observations implicate the potential of aldosterone blockade in the treatment of salt-associated cardiovascular disease. Although amiloride has repetitively been found effective on ECs both in vitro and ex vivo, its in vivo usage might be limited by the fact that a therapeutic concentration is only reached in the urine but not in the blood.\textsuperscript{39} Spironolactone, on the contrary, is well known to act directly on the vasculature.\textsuperscript{40} Its effectiveness in decreasing vascular stiffness and blood pressure in geriatric hypertension has already been demonstrated.\textsuperscript{41} Concomitantly, it was able to prevent vascular remodeling in old, normotensive rats\textsuperscript{42} and to inhibit the age-associated increase of proinflammatory markers in the vessel wall.\textsuperscript{21}

**Perspectives**

Aging is the major risk factor for cardiovascular disease. Given the current demographic development, a better understanding of the underlying pathogenic processes is necessary to provide affordable prophylaxis and therapy of these diseases and to meet the challenges the health system is going to face. The present experiments suggest that the aging process per se causes an increase in ENaC number in vascular endothelium and identify ENaC as a mediator of aging-related endothelial salt sensitivity. Considering the crucial role of vascular endothelium in vessel homeostasis, this might contribute to the development of systemic salt sensitivity and cardiovascular disease at age. The fact that aldosterone is required to render the endothelium sensitive to salt indicates the potential of aldosterone blockade in the prevention and treatment of cardiovascular disease. More research is required for the evaluation of this promising and elegant treatment strategy, specifically in the elderly.

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**Disclosures**

None.

**References**

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ONLINE DATA SUPPLEMENT

Endothelial sodium channels
trigger endothelial salt sensitivity with aging

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Short title: salt sensitivity with aging

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Expanded Materials and Methods

VE-cadherin immunostaining
Integrity of the EC monolayer after the process of preparation and cultivation was tested by immunostaining of the EC-specific junction protein VE-cadherin similarly as described in (1). Vessel patches were fixed with methanol (-20°C) for 20 min at 4°C. Unspecific antibody binding was blocked with 10% normal goat serum (NGS; Sigma Aldrich, Steinheim, Germany) for 30 min. The primary rat anti-mouse-CD144 antibody (eBioscience; San Diego, California, USA) was diluted 1:300 in NGS (10%) and applied to the patches for 1h at room temperature. The secondary donkey anti-rat antibody with a Alexa 488 fluorescent labelling (eBioscience) was diluted 1:500 in NGS (10%). Immunofluorescence images were acquired using an inverted fluorescence microscope (Axiovert 200, Zeiss, Oberkochen, Germany). Data acquisition was performed with the MetaMorph software (Molecular Devices, Sunnyvale, USA). A typical image is given in supplementary Fig. S1 (A).

Non-invasive blood pressure measurements
The CODA® non-invasive blood pressure system (Kent Scientific, Torrington, USA) is a computerized, non-invasive tail cuff acquisition system that utilizes a specially-designed differential pressure transducer to measure the blood volume in the tail. It records systolic arterial blood pressure (SAP), diastolic arterial blood pressure (DAP) and mean arterial blood pressure (MAP). 4 animals of each age group were practiced for 7 days while they were allowed free access to their diet and water. Readings were obtained between 13.00 h and 15.00 h after a slight warming on the 37°C warming plate. Multiple readings on individual mice were taken. To be accepted, measurements on a given mouse had to be virtually stable.

Reference List

Fig. S1
Fluorescence images of the endothelial cell monolayer in ex vivo aortae. A: Immunofluorescence staining of VE-cadherin shows that the EC monolayer is intact after the process of preparation and cultivation. B: DAPI staining of cell nuclei facilitates the location of the EC level. While EC nuclei are in focus, the characteristic pattern of vascular smooth muscle cell nuclei can be distinguished in the background.
Fig. S2
Continuous stiffness monitoring under normal sodium conditions. When HEPES buffered solution containing 135 mmol/L sodium was replaced by another HEPES buffered solution containing 135 mmol/L sodium, stiffness remained stable over the analyzed period of time. Thus, artefacts due to the solution exchange or continuous indentation could be excluded. n = 8
Fig. S3
Immunofluorescence images of QD-labeled α-EnNaC in the endothelial cell surface. Typical images are shown for the control group, the spironolactone group, the amiloride group and the negative control (no primary antibody). Top row: young mice; bottom row: old mice. It becomes obvious that under control conditions, endothelial cells of old mice exhibited significantly more EnNaC than those of young animals. Spironolactone and amiloride can be seen to reduce the number of channels in both age groups.