Role of Epithelial sodium channels (ENaCs) in endothelial function

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Abstract

An increasing number of mechano-sensitive ion channels in endothelial cells (ECs) have been identified in response to blood flow and hydrostatic pressure. However, how these channels respond to flow under different physiological and pathological conditions remains unknown. Our results showed that ENaCs were co-localized with hemeoxygenase-1 (HO-1) and hemeoxygenase-2 (HO-2) within the caveolae on the apical membrane of ECs and were sensitive to stretch pressure and shear stress. ENaCs kept low activities until their physiology environment was changed; in this case, the up-regulation of HO-1, which in turn facilitated heme degradation and hence increased the carbon monoxide (CO) generation. CO potently increased the bioactivity of ENaCs, releasing the channel from inhibition. Endothelial cells started to respond to shear stress by increasing the Na$^{+}$ influx rate. Elevation of [Na$^{+}$], hampered the transportation of L-arginine, resulting in impairing the nitric oxide (NO) generation. Our data suggested that ENaCs endogenous to human endothelial cells were mechano-sensitive. Persistent activation of ENaCs could inevitably lead to endothelium dysfunction and even vascular diseases such as atherosclerosis.

Key words: mechanical stress, ENaC, heme, NO, endothelium dysfunction
**Introduction**

The endothelium is a thin layer of cells that line the interior surface of entire blood vessels, forming a physical barrier between circulating blood elements and underlying tissues. Endothelial cells (ECs) are involved in many aspects of vascular biology, including the response to shear force, modulation of blood vessel tone and blood flow (Buchanan, Verbridge et al. 2014). There are many diverse responses of endothelial cells to hemodynamically related mechanical stress ranging from ion channel activation to gene regulatory events (Davies, Robotewskij et al. 1992).

Endothelial dysfunction is associated with most forms of cardiovascular diseases, such as hypertension (Antonello, Montemurro et al. 2007), coronary artery diseases (Goel, Majeed et al. 2007), peripheral artery diseases (Rhodes, Im et al. 2015), diabetes (Prattichizzo, Giuliani et al. 2015) and chronic renal failure (Johnson and Nangaku 2015). In hypertensive subjects with hyperaldosteronism, endothelium dependent flow-mediated vasodilatation is impaired (Nishizaka, Zaman et al. 2004). Increased pulmonary blood flow in immature animals produces endothelial cell dysfunction with loss of endothelium-dependent vasodilatation before the onset of pulmonary vascular remodeling (Vitvitsky, Griffin et al. 1998).

Ion channels expressed in ECs are considered to mediate ‘short-term’ responses (in a range of seconds and minutes) to shear stress and subsequently affect the cytoskeleton rearrangement, and the synthesis and/or release of pro- and anticoagulants, growth factors, and vasomotor regulators (Nilius and Droogmans 2001). ENaCs (epithelial sodium channels) are expressed in a variety of endothelial cell types (Wang, Meng et al. 2009). These channels play a central role in controlling Na$^+$ transport across epithelia and are thus of immense importance in all aspects of fluid clearance as well as numerous other functions. Administration of amiloride and benzamil, both antagonists of ENaCs, results in blockade of myogenic constriction of blood vessels (Jernigan and Drummond 2005), suggesting a potential role of ENaCs in mediating vascular tone. Changes in plasma [Na$^+$] are known to affect ECs function, suggesting that ENaCs expressed in endothelial may contribute indirectly to regulation of myogenic activity (Oberleithner, Riethmuller et al. 2007). Additionally aldosterone, a stimulator of ENaCs activity and translocation of ENaCs in EC membrane (Oberleithner, Riethmuller et al. 2006), has been shown to cause HUVEC swelling (Oberleithner, Schneider et al. 2003), endothelium stiffening (Jeggle, Callies et al. 2013) and NO production decreasing, resulting in endothelium dysfunction. Thus a role for endothelial ENaCs in control of vascular tone appears likely, but the nature of this role remains unclear. Additionally, inward rectified K channel (Kir) has long been considered the principal shear force-sensing ion channel in ECs since Kir is predominantly expressed in endothelial cells (Coleman, Tare et al. 2004) and the increase in Kir mediated K$^+$ conductance is one of the most immediate cellular response to shear force (Olesen,
Clapham et al. 1988). The primary cellular event following the activation of Kir eventually lead to the generation and release of NO, causing the underline SMC relaxation and hence vasodilatation (Nilius, Viana et al. 1997). In our previous work, CO has been shown to inhibit Kir (Liang, Wang et al. 2014). Thus, it seems that CO as the product of HO becomes a switch from Kir to ENaC. However, whether ENaC can specifically contribute to the flow sensing under stimulation of inflammatory factors is unknown and is addressed in this study.
Methods and materials

Cell culture and chemicals

Human umbilical vein endothelial cells (HUVECs) were freshly isolated from human umbilical cord vein (ethics approval by University Ethics Committee, Institute of Molecular Medicine, Peking University). HUVECs were grown in endothelial cells medium (Promocell) and maintained at 37°C and 5% CO₂. Cells were used within five passages.

All drugs were purchased from Sigma-Aldrich.

Electrophysiology recording

Single channel recordings were performed as previously described. Briefly, a coverslip or insert on which HUVECs had been cultured was transferred into a recording chamber mounted on a Nikon inverted microscope (Nikon TE 2000U). Patch pipettes of resistance 6 MΩ were fabricated from borosilicate glass capillaries (1.5 mm od, 0.86 mm id; Sutter) using a Sutter P97 pipette puller. Bath solution contained (in mM): 110 NaCl, 4.5 KCl, 1 MgCl₂, 1 CaCl₂, 5 HEPES, 5 Na-HEPES, pH 7.2. Pipette solution contained (in mM): 110 NaCl, 4.5 KCl, 0.1 EGTA, 5 HEPES, 5 Na-HEPES, pH 7.2. In the study of inward rectified potassium channels, bath solution contained 140 NaCl, 5.4 KCl, 1.8 CaCl₂, 1 MgCl₂, 5 HEPES, 10 glucose, pH 7.4 (adjusted with NaOH) and the pipette solution contained (in mM): 100 potassium aspartate (Sigma-Aldrich), 30 KCl, 1 MgCl₂, 5 HEPES, 5 EGTA, 4 K2-ATP, pH 7.3 (adjusted with KOH).

Single channel currents were recorded with an Axon 200B amplifier connected to a PC running Axon clampex 9.0. The data were acquired at 20 KHz and low pass filtered at 5 kHz. During offline analysis, data were further filtered at 200 Hz. Single channel events were listed and analyzed by pclampfit 9.0 (single channel search in analyze function). 50% threshold cross method was utilized to determine valid channel openings. When multiple channel events were observed in a patch the total number of functional channels (N) in the patch was determined by observing the number of peaks detected on all points amplitude histograms. NPo, the product of the number of channels was used to measure the channel activity within a patch. Initial, 3-4 minute, single channel records were normally used as the control. The activity of ENaC during application of chemicals was normalized to activity during the control period to assess the effects of chemicals on ENaC activity. In some cases ENaC activity during application of chemicals was also compared to that of ENaC when chemicals were washed off. These data were used to confirm the effects of chemicals. Data are presented as means±SEM. Means were compared using Student’s paired t test. Statistical significance was set as < 0.05, represented on figures as *.

Whole cell recording was carried out using pipettes of 6 MΩ resistance as detailed above. Bath sa-
line was as described above but with the addition of 10mM D-glucose. The pipette solution contained (in mM): 40 KCl, 100K-gluconate, 1 MgCl₂, 1 CaCl₂, 0.1 EGTA, 4 Na₂ATP, 10 Glucose, 10 HEPES, 2 GTP and pH was adjusted to 7.2 with KOH. For perforated patch whole-cell recording, the pipette solution was supplemented with 10-20μg/ml amphotericin B. On the day of experiment, 0.1 g amphotericin B (Sigma) was weighted and dissolved in 0.1-0.5ml DMSO to make a stock solution. 0.5μl of this stock solution was added into 1 ml of pipette solution to make up final pipette solution, giving a final amphotericin B concentration of 10-20 μg/ml. DMSO (vehicle for amphotericin B) at the same dilution was tested in control experiments but no effect was observed.

**Flow setup**

Flow setup were performed as described by David E. Clapham with modifications (Oancea, Wolfe et al. 2006). A pipette with a wide opening in a range of 100-200 μm which was connected to a syringe pump (Harvard Apparatus, Harvard) was placed within 160 μm of the interested cell. When whole cell configuration was obtained and cell membrane potential was clamped at -100 mV, the pump was switched to generate a relative laminar flow over the interested cell. Flow-induced shear force applied to the interested cell was calculated according to the equation:

\[ T = \frac{\rho V^2}{2} \times \frac{0.664}{\sqrt{R_x}} \]

\( \rho \) is the density of water (1025kg/m³)

\( V \) is the fluid velocity (calculated using \( V = \frac{Q}{A} \), where \( A = \pi d^2/4 \) (d is the flow application pipette diameter) and \( Q \) is the flow rate generated by the syringe pump measured in m³/s

\( R_x = V \times X / \mu \) where \( X \) is the distance between pipette and cell, \( \mu \) is the kinematic viscosity of the water (1.139 X 10⁻⁶m²/s)

In our setup, d was approximately 120 μm and X approximately160 μm. Accordingly, 1 ml/min of flow generated 0.49 dyne stress; 3 ml/min of flow was 3.67 dyne; 5 ml/min was 8.42 dyne; 8 ml/min was 17 dyne.

All data were obtained consistently. Whole cell recording is a perfect way to monitor and valuate the channel response to shear force. Therefore, it is occupied in the study of shear force response of ENaCs. However, cell-attach recording/single channel recording is a good way with less bias to measure the response to hydrostatic pressure and was occupied in the study of mechanical response of ENaCs. Of 10mmHg hydrostatic pressure were then applied in this section of study to uniform stimuli.
Isolation of caveolae compartment and western blot

HUVECs were grown to confluence in 75ml culture flasks and used for fractionation. The homogenates in MBS (25 mM 2-(N-morpholino)-ethanesulfonic acid (MES), pH 6.5, 0.15 M NaCl) containing 1% Triton X-100 were adjusted to 40% sucrose by the addition of 2 ml of 80% sucrose prepared in MBS and placed at the bottom of an ultracentrifuge tube. A 5–30% discontinuous sucrose gradient was formed above (4 ml of 5% sucrose/4 ml of 30% sucrose, both in MBS lacking detergent) and centrifuged at 39,000 rpm for 18 h in an SW41 rotor (Beckman Instruments, Palo Alto, CA, USA). A light-scattering band at the 5–30% sucrose interface was collected or fractionated into 12 sub-fractions.

Each protein sample was loaded and separated on 10% Bis-Tris Gel. The membranes were then blocked with 5% non-fat dry milk, probed with appropriate primary antibodies, followed incubation by HRP-conjugated secondary antibodies at a dilution of 1:3000. The following antibodies were used: α-ENaC antibody (Santa Cruz, sc-21012), HO-1 antibody (Santa Cruz, sc-1796), HO-2 antibody (Proteintech, 14817-1-AP) and caveolin-1 antibody (Proteintech, 1644-1-AP).

Cell Transfection

The HUVECs were seeded and one day after seeding, HUVECs were transfected with siRNA against HO-1 (GenePharma, Shanghai, China). In short, siRNA was mixed with PromoFectin-HUVECs (Promocell) in Opti-MEM reduced serum medium (Invitrogen) and incubated for 20 min at room temperature before being transferred to the apical side of the monolayer and further incubated for 5 h at 37 °C. The transfection medium was then replaced with endothelial cells medium without antibiotics. Knockdown effects were examined by western blots analysis after 24 hours. The sequences targeted to silence HO-1 mRNA were:

5’-GGGAAUUUAUGCCAUGUAATT-3’(sense), 5’-UUACAUGGCAUAAAUCCCTT-3’(antisense).

Measurement of Endothelial Nitric Oxide Production

HUVECs were stimulated by shear force for 8 hours with the treatment of benzamil and amiloride. Then ECs were incubated with 5μM DAF-FM (Biyuntian, China) diacetate in phenol red-free DMEM for 30 min at 37 °C. The cells were washed gently with PBS for 3 times, DAF fluorescence was recorded by 60× oil objective lenses and analyzed with laser scanning confocal microscopy (Nikon).
**Tension studies of aorta rings**

Aorta tension studies were carried out in a manner as previously published with modifications (Faury, Ristori et al. 1995). Male Wistar rats (250g) were anaesthetized with sodium pentobarbital (55 mg/kg, ip) and killed by cervical dislocation. Rat aorta was quickly removed to a bath containing cold physiological salt solution (PSS) for dissection (in mM: NaCl 154.7, KCl 5.4, D-glucose 11.0, CaCl2 2.5, Tris 6.0, pH 7.4). And the distal aorta was dissected free of surrounding tissue carefully, cut as rings and mounted in a temperature controlled myograph system (DanisMyo Technology A/S model: 610 M). The bath solution (PSS) was gassed with 100% O₂ at 37°C. Each ring was initially stretched to give an optimal pressure of 200g and the preparation was allowed to stabilize for 60 min. 10uM acetylcholine and 20ug/ml LPS were used to perfuse. Tension data was relayed from the pressure transducers to a signal amplifier (600 series eight-channel amplifier, Gould Electronics). Data were acquired and analysed with Pclamp software.

**Statistical analysis**

The Student's t-test was used for the statistical analysis of all the independent experiments, with significance accepted at $P<0.05$. In the patch-clamping assays, data from one coverslip were averaged and presented in the figures. The same experiment was repeated on different coverslips (N). Data were presented as mean ± SEM, and statistical differences were assessed using the Student’s paired t-test. $P<0.05$ was considered significant.
Results

1. ENaCs were activated by fluid flow.
We used whole cell patch-clamping to record the Na\(^+\) currents (Fig.1A). A flow rate at 5 ml/min (equivalent to 8.42 dyne) significantly augmented inward currents (Fig.1B). This effect was completely reversed by benzamil (10μM), an antagonist of ENaC (Fig.1B). This indicated that the shear-induced inward current was due to activation of ENaCs. The potentiation of inward current (normalized to control current) by fluid flow was directly dependent upon the amplitude of the flow-induced shear force, saturating at approximately 12 dyne (Fig.1C).

2. ENaCs were activated by hydrostatic pressure and stretch.
Positive pressure rather than suction was applied to the pipette, thus mimicking the effects of hydrostatic pressure on the apical surface of the membrane. Application of 10 mmHg increased activity of ENaCs almost 2.5 fold (Fig.2A; P<0.05). Channel activity reached a maximum within 1s of pressure application and decayed over a period of several seconds after release of pressure. Repeated stimulation elicited a similar response on every application (Fig.2A). Intriguingly, the flow-induced increase of channel open probability was also present in a cell-attach recording configuration where target channels have in fact no direct contact with flow (Fig.2B), suggesting that ENaCs can be, at least, activated by cell membrane tension and curvature.

3. Regulation of ENaCs by Heme and CO.
In the inside out configuration with a pipette voltage of +80 mV, we obtained the traces. Different concentration of heme could inhibit the ENaCs activity (Fig.3A a) and the inhibitory effects were in a dose-dependent manner (Fig.3B). However, heme plus NADPH could activate ENaCs activity compared to that only added heme (Fig.3A b). This effects were reversed when knockdown HO-1 (HO-1\(^-\)) expression (Fig.3A c,d & 3C). CO, the production of heme plus NADPH also could activate ENaCs activity (data not shown).

4. ENaCs were co-localized with caveolin-1, HO-1 and HO-2.
Previous reports have demonstrated that ENaCs are present within lipid rafts (Sagi-Eisenberg, Lie-man et al. 1985). Accordingly, HUVECs were lysed and the components of the membrane separated by its density in the sucrose gradients. Twelve fractions were obtained; each fraction was precipitated and then immune-blotted with antibodies against the α-subunit of ENaC, caveolin-1, HO-1 and HO-2. Low density membranes which correspond to lipid rafts were found near the top of the gradient in fractions of 4 and 5, while non-raft markers were found at higher sucrose densities in frac-
Caveolin-1 was found in fractions 4 and 5. As well as ENaC α-subunit, HO-1 and HO-2 were detected in these fractions (Fig.4A), suggesting ENaC, HO-1 and HO-2 were co-localized with caveolin-1.

5. **TPA-induced increase in HO expression enhances ENaCs activity and shear sensitivity.**

TPA (phorbol 12-myristate 13-acetate) is a potent tumor promoter and also regarded as a common inflammatory factor (DeRiemer, Strong et al. 1985). TPA at a concentration of 100ng/ml was added into the growing medium 24 hours prior to experiments. Western blot analysis showed that HO-1 expression was significantly increased after 24 hours stimulation of TPA, whereas caveolin-1, HO-2 and α subunit of ENaCs remained unchanged (Fig.4B).

We then performed perforated whole-cell patch clamp, a non-invasive method which monitors the whole-cell currents while keeps the intracellular signal molecules operative. Perforated whole-cell patch clamping results demonstrated a significant increase in Na+ conductance in response to flow rate at 3 ml/min (equivalent to 3.67 dyne), which is negligible in the results acquired from the non-TPA treated HUVEC cells under same conditions (Fig.4C). Additionally, TNF-α (25ng/ml) exerted the similar stimulation effects as TPA (data not shown). Taking all these data together, we conclude that the inflammatory factors could enhance ENaCs activity and shear sensitivity.

6. **Increasing ENaCs activity impeded cationic amino acid transporter (CAT) activity and NO production.**

Increased ENaCs activity leads to the elevated \([Na^+]_i\) in the endothelial cells. We used whole-cell patch clamping to examine the corresponding currents induced by cationic amino acid transporter (CAT) under different concentration of \([Na^+]_i\). The inward currents evoked by extracellular L-arginine were significantly reversed by increase of \([Na^+]_i\) in a manner of dosage dependence (Fig.5A). The cellular availability of L-arginine is decided by the transport capacity of CAT. Increased \([Na^+]_i\) as we have demonstrated, reduces CAT driving force and in turn impairs the L-arginine entry. Therefore, ENaCs mediated shear force-sensing is likely to exert a reverse effect on EC functions as it impairs the endothelial-dependent NO generation. We treated HUVECs with shear force and found that NO production decreased due to the activation of ENaCs by shear force, while the antagonists of ENaCs, benzamil and amiloride could reverse the process (Fig.5B). Next, we isolated the aorta from the Wistar rats and explored the tension of aorta rings. The data shown that LPS (20ug/ml) failed to dilate vessels due to induced elevation of CO and consequently ENaC activation, while the acetylcholine (10uM) stimulating NO release could promote the vasodilatation (Fig.5C).
Discussion

We have identified the expression and the mechano-sensitivity of ENaCs in endothelial cells, determined the up-regulation of HO-1 expression by inflammatory factors, and validated the effects of the HO-1 mediated heme degradation pathway on ENaCs. By piecing all these findings together, a scenario appeared. ENaCs are expressed on endothelial cells, and this expression not only retains the channel function as a Na\(^+\) entry pathway but also possesses mechano-sensing ability. However, ENaCs were kept silent until their physiology environment changes; in this case, the appearance of inflammation. Pro-inflammatory factors greatly up-regulate the highly inducible protein HO-1, which in turn facilitates heme degradation and hence increases the CO generation. CO potently increases the bioactivity of ENaCs, releasing the channel from inhibition, while on the other hand exerts a strong inhibitory effects on Kir channels (Liang, Wang et al. 2014). This aspect of CO impact on ECs can be therefore described as that CO shifts the duty of mechano-sensing from Kir to ENaC. Eventually, endothelial cells start to respond to shear stress by increasing the Na\(^+\) influx rate. Elevated [Na\(^+\)], impairs the L-arginine entry, resulting in impairing the nitric oxide (NO) generation, while benzamil and amiloride, both the antagonists of ENaCs, could rescue NO production. Thus, it is predictable that long-term ENaC hyper-activation induced by chronic inflammation will lead to a decreased NO generation in endothelial cells and the vasoconstriction.

Our results are consistent to many previous clinical reports (Nishizaka, Zaman et al. 2004). For example, a decreased L-arginine conversion to NO has been shown in these hypertensive patients after salt loading (Ni and Vaziri 2001). Flow-mediated vasodilatation (FMD) was significantly reduced in patients compared with healthy control subjects (Pemp, Weigert et al. 2009). Heme oxygenase (HO) is the rate-limiting enzyme in heme catabolism, which leads to the generation of CO (Maines 1997). HO and its metabolic products carbon monoxide (CO) have been implicated in regulation of basal tone and blood pressure (Johnson, Lavesa et al. 1996). CO is a promising molecule with therapeutic potential in a number of vascular disorders due to its cytoprotective and homeostatic properties (Dulak, Loboda et al. 2008). Carbon monoxide inhibits sprouting angiogenesis and vascular endothelial growth factor receptor-2 phosphorylation (Ahmad, Hewett et al. 2015). Carbon monoxide releasing molecule (CORM-2) could promote endothelial repair, and inhibit neointima formation after carotid artery balloon injury (Hu, Chen et al. 2015).

Many studies have shown that relationship between NO and HO-1. NO and CO are both gaseous messengers are employed for intra- and intercellular communication with high specificity. It has been suggested that CO and NO could be bound to heme-containing proteins exert its regulation (Ding, McCoubrey et al. 1999). It remains unclear the nature of CO receptor with exception of NO and guanylyl cycles (GC) (Leffler, Parfenova et al. 2006). NO can affect HO-2 catalytic activity
which produces CO either inhibitory or augmentory (Ding, McCoubrey et al. 1999). In isolated heart (Maulik, Engelman et al. 1996) and porcine aortic endothelial cells, NO increase CO production. NO may have a direct inhibitory effect on HO-2 that is masked in the intact system by cGMP-induced stimulation. Alternatively, extravascular sources of CO in vivo may contribute to pial arteriolar dilation to glutamate. In the isolated microvessels NO synthase (NOS) inhibition totally abolished glutamate-induced CO production (Leffler, Balabanova et al. 2005). Additionally, CO is much less effective at simulating GC than is NO (Kim, Wang et al. 2005). Normal dose-dependent dilation to CO occurs with cGMP held constant. CO may exert its effect via its activation of BK$_{Ca}$ whereas NO also can affect BK$_{Ca}$ channels via protein kinase G (Leffler, Nasjletti et al. 1999).

Once the endothelium-dependent NO production is hampered, vessels start to lose their ability to mitigate increased blood pressure by dilatation, which further deteriorate the EC dysfunction or even cause cell damage. Moreover, decreased NO bioactivity leaves vessels unprotected and vulnerable to platelets aggregation and adhesion molecules expression (Mury, Brunini et al. 2014). Eventually, the initiation and progression of vascular diseases such as atherosclerosis is inevitable. Therefore, these results in fact identified a pathological pathway that leads chronic inflammation to endothelial cell dysfunction characterized by a decreased NO bioactivity, which is considered the first step towards vascular diseases.

Our data identify that ENaCs endogenous to human endothelial cells are mechano-sensitive. Persistent activation of ENaCs induced by stimulation of inflammatory factors will inevitably lead to endothelium dysfunction and hypertension.
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Conflicts of interest
Authors disclose there is no conflict of interest.
References:


**Figure 1. Flow activated ENaCs.**

A. In a conventional whole cell recording, ENaCs currents were elicited by a voltage-ramp protocol.

B. In a conventional whole cell recording, flow at a speed of 5 ml/min, which equivalent to 8.42 dyne, significantly enhanced the inward currents mediated by ENaCs, which was reversed and blocked by benzamil (10μM). Each point represented the mean ± SEM, P<0.05, n=6-8.

C. Flow at a speed of 1.3 and 5 ml/min, which equivalent to 0.49, 3.67 and 8.42 dyne, significantly enhanced the inward currents mediated by ENaCs in a dose-dependent manner, which was reversed and blocked by stop flow. X axis represented shear stress against on cell membrane and y axis represented the increase ratio of whole cell current density carried by ENaCs. Each point represented the mean ± SEM, n=4-5.
Figure 2. Stretch activated ENaCs.

A. In an inside-out recording, positive pressure (10 mmHg) via the patch pipette on outside membrane of HUVECs activated ENaCs currents in a repeatable manner. The pipette voltage was held at 80mV. Stretch significantly increased ENaCs activity by 1.5 folds. Each point represented the mean ± SEM, n=8 (right side).

B. Flow indirectly activated ENaCs. In a cell-attach recording, flow significantly increased the open probability of ENaCs, suggesting the present of an indirect mechanism. Application of pressure was shown by the grey bars above the upper current trace. Selected parts of currents (marked by bars below the upper trace) was shown with an expanded time scale in the lower panel as in the numeric traces. Each point represented the mean ± SEM, n=5.
Figure 3. Heme alone inhibited ENaCs whereas Heme plus NADPH activated ENaCs in HU-VECs.

A. Traces were obtained in the inside out configuration with a pipette voltage of +80 mV.
   a) showed different concentration of heme inhibit ENaCs activity.
   b) showed that heme plus NADPH (1μM) activated ENaCs.
   c) showed that heme plus NADPH (1μM) while HO\textsuperscript{-1} knockdown (HO\textsuperscript{-1–}) inhibited ENaCs.
   d) showed the western blots effects of HO-1 knockdown.

B. Concentration dependent effects of heme or heme plus NADPH on ENaCs activity. Data were fitted with a sigmoidal dose-effect curve. Hill coefficient is 0.99, based on the equation: 
   \[ Y = \text{minimum} + (\text{maximum} - \text{minimum})/(1 + (X/X0)^p) \]. Blue line with black square symbols represents effect of heme alone. Each point represented the mean ± SEM, n=6-8.

C. Heme (20nM) significantly inhibited ENaCs activity whereas in presence of heme (20nM) plus NADPH, ENaCs from HUVECs with knockdown of HO-1 were inhibited. Each point represented the mean ± SEM, n=10.
Figure 4. TPA facilitated flow response mediated by ENaCs in HUVECs.

A. Caveolae compartmentalization of ENaCs, HO-1 and HO-2 in HUVEC. Total cell lysate was loaded on the discontinuous sucrose gradients for 18 hours (39,000 rpm, SW41 rotor). 12 fractions of each were obtained and subjected to Western blot analysis. Caveolin-1 was concentrated in fractions 4-5, where ENaCs, HO-1 and HO-2 were also present.

B. Incubation HUVEC with TPA (100ng/ml) in medium significantly augmented HO-1 expression but not HO-2 and ENaCs in 24 hours.

C. TPA treatment significantly enhanced ENaCs whole cell currents and flow response mediated by ENaCs. Flow at a speed of 3ml/min, which equivalent to 3.67 dyne. Each point represented the mean ± SEM, n=10.
Figure 5. Increase of ENaCs activity impeded cationic amino acid transporter (CAT) activity and NO production.

A. Increase of [Na⁺]ᵢ attenuated the activity of CAT. The inward currents evoked by extracellular L-arginine were significantly reversed by increase of [Na⁺]ᵢ in a manner of dosage dependence. Each point represented the mean ± SEM, n=4-6. The curve was fitted by Exp-decay (first) on the equation y=A₁*exp(-x/t₁)+y₀.

B. Shore force decreases the NO production in HUVECs and the antagonists of ENaCs, benzamil and amiloride could reverse the process. Results represented the mean ± SEM, n=5. Scale bar=10 µm.

C. The tension of aorta rings. Acetylcholine (10uM) stimulating NO release promoted the vasodilatation, while LPS (20ug/ml) failed to dilate vessels due to induced elevation of CO and consequently ENaCs activation.