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Original Paper

Elevation of Intracellular Na^+ Contributes to Expression of Early Response Genes Triggered by Endothelial Cell Shrinkage

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Key Words
Endothelium • Cell shrinkage • Ouabain • Intracellular Na^+ • Early response genes • Transcription

Abstract
Background/Aims: Prolonged hyperosmotic shrinkage evokes expression of osmoprotective genes via nuclear factor NFAT5-mediated pathway and activates Na^+ influx via hypertonicity-induced cation channels (HICC). In human umbilical vein endothelial cells (HUVEC) elevation of intracellular sodium concentration ($[Na^+]_i$) triggers transcription of dozens of early response genes (ERG). This study examined the role of monovalent cations in the expression of Na^+_i-sensitive ERGs in iso- and hyperosmotically shrunken HUVEC. ***Methods:*** Cell volume was measured by 3D reconstruction of cell shape and as ^{14}C-urea available space. Intracellular Na^+ and K^+ content was measured by flame atomic absorption spectrometry. ERG transcription was estimated by RT-PCR. ***Results:*** Elevation of medium osmolality by 150 mM mannitol or cell transfer from hypo- to isosmotic medium decreased cell volume by 40-50%. Hyperosmotic medium increased $[Na^+]_i$ by 2-fold whereas isosmotic shrinkage had no impact on this parameter. Hyperosmotic but not isosmotic shrinkage increased up-to 5-fold the content of EGR1, FOS, ATF3, ZFP36 and JUN mRNAs. Expression of these ERGs triggered by hyperosmotic shrinkage and Na^+,K^+-ATPase inhibition by 0.1 μM ouabain exhibited positive correlation (R^2=0.9383, p=0.0005). Isosmotic substitution of NaCl by N-methyl-D-glucamine abolished an increment of $[Na^+]_i$ and ERG expression triggered by mannitol addition. ***Conclusion:*** Augmented expression of ERGs in hyperosmotically shrunken HUVEC is mediated by elevation of $[Na^+]_i$.

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Introduction

Animal cells lacking solid envelope behave as an osmometer and change their volume in response to any stimuli affecting the osmolality of extracellular fluids or/and cytoplasm. Cell volume changes occur in the proportion to the volume of intracellular water that, in turn, increases cytoplasmic macromolecular crowding affecting functions of diverse proteins [1]. In distal renal tubules osmolality of luminal fluid varies from 100 to 4000 mOsm [2]. Recent studies demonstrated that high-salt diet leads to sustained elevation of osmolality of cerebrovascular [3] and subcutaneous interstitial fluids [4, 5].

It is well documented that prolonged cell swelling and shrinkage in anisosmotic media affects expression and/or activity of genes involved in the triggering and progression of two morphologically distinct modes of cell death, i.e. necrosis and apoptosis, respectively (for review, see [6-10]). To escape the cytotoxic effects of prolonged volume perturbations, cells maintain their volume with 1-3% accuracy via systems providing regulatory volume decrease (RVD) and regulatory volume increase (RVI). Rapid RVD and RVI are triggered in several seconds after cell swelling and shrinkage via activation of channels and carriers involved in accumulation or loss of monovalent ions, respectively [11-13]. It should be noted, however, that rapid cell volume autoregulation via activation of ion transporters is accompanied by elevation (RVI) or attenuation (RVD) of cytoplasm ionic strength resulting in drastic modulation of macromolecular complexes activity [6, 12, 14]. To protect from these side effects of rapid volume autoregulation, cells exposed to chronically elevated osmolality accumulate organic osmolytes via augmented expression of SMIT, BGT1 and TauT, i.e. genes providing Na^+-coupled accumulation of inositol, betaine and taurine, respectively. In several types of cells, hyperosmotic environment also results in accumulation of sorbitol and glycerolphosphocholine via augmented transcription of aldoreductase (AR) and neuropathy target esterase (NTE), respectively [2, 15, 16]. It was shown that augmented transcription of SMIT, BGT1, TauT and dozens of other genes is mediated by tonicity responsive enhancer (TonE) containing several NGGAAAWDHMC(N) repeats and located within proximal sites of 5'-promoters. TonE is activated by protein termed as tonicity-responsive enhancer-binding protein or osmotic-response element-binding protein (TonEBP/OREBP). Using yeast one-hybrid screening and homology cloning strategies TonEBP was identified as NFAT5, i.e. a member of superfamily of nuclear factor of activated T cells proteins (for review, see [17-19]).

Using Na^+- and K^+-sensitive fluorescent dyes Wehner and co-workers found that in cultured rat hepatocytes elevation of medium osmolality from 300 to 450 mOsm resulted in rapid rise of $[Na^+]_i$ and $[K^+]_i$ from 15 to 30 mM and from 80 to 110 mM, respectively [20]. Later on the same research team reported that in human hepatoma HepG2 cell line this phenomenon is caused by activation of hyperonicity-induced cation channels (HICC) permeable for Na^+, K^+, Cs^+ and Li^+ and inhibited by 10 μM amiloride, 100 μM Gd^{3+} and 10 μM flufenamate [21, 22]. They also demonstrated that activation of HICC rescues staurosporine-treated HeLa cells from apoptosis [23]. It was shown that hypertonicity-induced monovalent ion fluxes might be mediated by α-subunit of epithelial Na^+ channel ENaC and transient receptor potential cation channel TRPM2 [24, 25]. More recently, we compared the action of ouabain and K^+-free medium, i.e. two independent approaches for Na^+,K^+-ATPase inhibition, on the transcriptomic changes in human adrenocarcinoma HeLa cell line, rat vascular smooth muscle cells and human umbilical vein endothelial cells. In these studies, we found 80 ubiquitous transcripts whose expression was increased up-to 20-fold under sustained dissipation of the transmembrane gradient of monovalent cation and elevation of the $[Na^+]_i/[K^+]_i$ ratio including augmented content of mRNAs encoding interleukin-6 (IL-6), cyclooxygenase-2 (COX-2 or PTGS2) and serum and glucocorticoid-regulated kinase 1 (SGK1) [26-28], i.e. genes whose augmented expression was observed in renal epithelial cells, macrophages, hepatocytes and endothelial cells subjected to hyperosmotic shrinkage [29-34]. Viewed these data collectively we proposed that together with TonEBP, altered gene

expression in shrunken cells might be caused by Na^+_i,K^+_i-sensitive mechanisms of excitation-transcription coupling [35]. The present study examines this hypothesis.

Materials and Methods

Cell culture

Human umbilical vein endothelial cells (HUVEC) were purchased from Lonza (Walkersville, MD, USA) and passaged 4-8 times. The cells were cultured in complete endothelial cell growth medium-2 (EGM-2 BulletKit, CC3162, Lonza) containing 10% fetal bovine serum (FBS) and maintained in a humidified atmosphere with 5% CO_2/balance air at 37°C. To establish quiescence, the cells were incubated for 24 h in medium in which the concentration of FBS was reduced to 0.2%.

Cell shrinkage protocols

The cells were washed twice with 2-ml aliquots of medium containing 150 mM NaCl and 10 mM HEPES-tris buffer (pH 7.4, 37°C), then incubated for 30 min in 0.5 ml of isosmotic (293 mOsM) medium A containing 135 mM NaCl, 3.8 mM KCl, 1.2 mM $MgSO_4$, 1.3 mM $CaCl_2$, 1.2 mM KH_2PO_4, 10 mM D-glucose, 10 mM HEPES (pH=7.4, adjusted with NaOH), isosmotic Na^+-free medium B (the same as medium A but NaCl was substituted for N-methyl-D-glucamine (NMDG, final osmolality 302 mOsM) or hyposmotic (197 mOsm) medium C (the same as medium A but NaCl concentration was reduced to 85 mM). Hyperosmotic shrinkage was caused by the addition in isosmotic media A or B the equal volume of the same medium containing 300 mM mannitol (final medium osmolarity of 435 mOsm). Isosmotic shrinkage was triggered by adjusting isosmotic conditions in cells preincubated in hyposmotic medium C the same medium containing 185 mM NaCl (final NaCl concentration 135 mM). Medium osmolarity was measured with Knauer osmometer (Berlin, Germany). In part of experiments, cells were incubated in medium A containing ouabain at concentrations indicated in the table and figure legends.

Cell viability

CTS impact on cell viability was studied by cell attachment assay as described in detail elsewhere [36]. Briefly, the HUVEC were seeded into 6-well plates and treated as indicated above. Then, the incubation medium was transferred into centrifugation tubes and combined with medium obtained after three washes in 2 ml of PBS. The detached cells were sedimented (1, 000 g, 5 min) and washed once with 5 ml of PBS. The protein content of detached cells (PR_{det}) and cells attached to the plastic supports after three washes with 2-ml aliquots of medium W (PR_{att}) was measured by a modified Lowry method. Total protein content (PR_{att} + PR_{det}) was taken as 100%. In additional experiments, cell viability was assessed by measuring caspase-3 activity and chromatin cleavage. Caspase-3 activity in cells growing in 6-well plates was measured as the rate of the caspase-3 inhibitor (Ac-DEVD-CHO)-sensitive component of caspase-3 fluorescent substrate (DEVD-AMC, N-acetyl-Asp-Glu-Val-Asp-AMC) hydrolysis according to previously described protocol [37]. To estimate chromatin fragmentation, HUVEC in 24-well dishes were supplied with DMEM containing serum and 0.1 μCi/ml [^{3}H]-thymidine. After 24 h, they were washed twice with 2 ml of DMEM and incubated for 48 h in DMEM with serum and compounds as indicated in the figure and table legends. Then the medium was collected and centrifuged at 900 g for 10 min. Next, the supernatant was transferred for the measurement of radioactivity in a liquid scintillation spectrometer (fraction F_1), and the cell pellet and cells remaining in the plates were treated for 15 min with ice-cold lysing buffer (10 mM EDTA, 10 mM Tris-HCl, 0.5% Triton X-100, pH 8.0). Then the cell lysates were combined, sedimented (12, 000 rpm, 10 min), and the supernatant was transferred for radioactivity measurement (fraction F_2). The remaining radioactivity from the pellets and wells was extracted with a 1% SDS/4 mM EDTA mixture (fraction F_3). The relative content of intracellular chromatin fragments was determined as a percentage of total [^{3}H]-labeled DNA: $F_2/(F_1+F_2+F_3)^{-1}$ x 100%. For more details, see [37].

Cell volume. Cell volume was measured in substrate-attached cells with an improved version of the DISUR technique [38]. This method involves 3D reconstruction of cell shape based on 2 conventional microscopy cell images acquired in 2 perpendicular directions. Side-view and top-view cell images were captured by 2 independent, miniature, charge-coupled cameras (Moticam 350, Motic Instruments Inc., Richmond, BC, Canada) with Motic software at 10- to 60-s intervals to closely follow rapid volume changes.

The images served to generate a set of topographical curves of the cell surface from its digitized side-view profile and base outline. Cell volume was calculated from the reconstructed cell topographical model with MATLAB (Math Works, Inc., Natick, MA). For more details, see [39, 40].

Intracellular water volume. Intracellular water volume in cells seeded in 12-well plates was measured as [^{14}C]-urea available space according to a previously-described protocol [41] and calculated as $V = A_c / A_m m$, where A_c was the radioactivity of the cells after 30-min incubation with 2 μCi/ml [^{14}C]-urea (dpm), A_m was the radioactivity of the incubation medium (dmp/μl), and m was protein content in the cell lysate (mg).

Intracellular Na^+ and K^+ content

Six-well plates were transferred onto ice, experimental medium was quickly removed, and cells were washed three times with 3 ml of ice-cold 0.1 M $MgCl_2$ solution in doubly deionized (DDI) water. Then, 1.5 ml of 5% trichloroacetic acid (TCA) in DDI water was added to each well, followed by incubation at 4°C overnight for complete extraction of ions from cells. Cell precipitates were suspended and centrifuged for 5 min at 18, 000xg. Supernatants were transferred to test tubes and stored at –20°C. Cell precipitates were resuspended in 0.75 ml of 0.1 M NaOH and incubated at 65°C for 1 h for complete protein dissolution. The resulting protein solutions were used for protein quantification by the Lowry protein assay [42]. The Na^+ and K^+ contents in TCA extracts were measured by flame atomic absorption spectrometry using a Kvant-2m1 spectrometer (Cortec, Russia) with propane-air mixture in accordance with the manual. Solutions of KCl (0.5-4 mg/liter K^+) and NaCl (0.05-2 mg/liter Na^+) in 5% TCA in DDI water were used for calibration. The Na^+ and K^+ contents in each well were normalized to protein amount in the same well. For more details, see [43].

Real-time quantitative RT-PCR

To examine transcription of selected genes, we performed qRT-PCR using Express SYBR GreenER qPCR Supermix kit (Invitrogen, Carlsbad, CA, USA) according to the manufacture's instructions. The reaction was carried out with a 7900 HT Fast RT – PCR system (Applied Biosystems, Foster City, CA, USA). Primers were designed using Primer3Plus online software from consensus sequences provided by Affymetrix for each gene of interest.

The relevant primer sequences were:

EGR1 (early growth response 1)

forward: GCACCTGACCGCAGAGTCT, reverse: AGATGGTGCTGAGGACGAGG;

FOS (FBJ osteosarcoma oncogene)

forward: CTGGCGTTGTGAAGACCAT, reverse: TCCCTTCGGATTCTCCTTTT;

ATF3 (activating transcription factor 3)

forward: CGTGCTGCTCTACGACATGA-3', reverse: GCTCCAACTGAAGGTCCCTG

ZFP36 (zinc finger protein 36)

forward: CATGGCCAACCGTTACACC, reverse: AGCGACAGGAGGCTCTCGTAC;

JUN (Jun oncogene)

forward: GCATGAGGAAACGCATCGCTGCCTCCAAGT; reverse: GCGACCAAGTCCTTCCCACTCGTGCACACT.

All experiments were analyzed in duplicate. β_2 microglobulin mRNA expression was used to normalize and compare the expression values of genes of interest. The results were quantified by the ΔΔCt method with Excel Microsoft software.

Chemicals

[^{14}C]-urea was obtained from PerkinElmer (Waltham, MA, USA). DEVD-AMC, DEVD-CHO and z-VAD. fmk were procured from BIOMOL Research Laboratories (Plymouth Meeting, PA). The rest of chemicals were obtained from Merck (Germany), Helicon and Reachem (Russia) and Sigma-Aldrich (St. Louis, MO).

Results

Effect of hyper- and isosmotic shrinkage on cell volume and intracellular Na^+ and K^+ content

Fig. 1 shows that elevation of medium osmolality by the addition of 150 mM mannitol lead to rapid ~50% attenuation of cell volume estimated by DISUR technique (Fig. 1). Similar

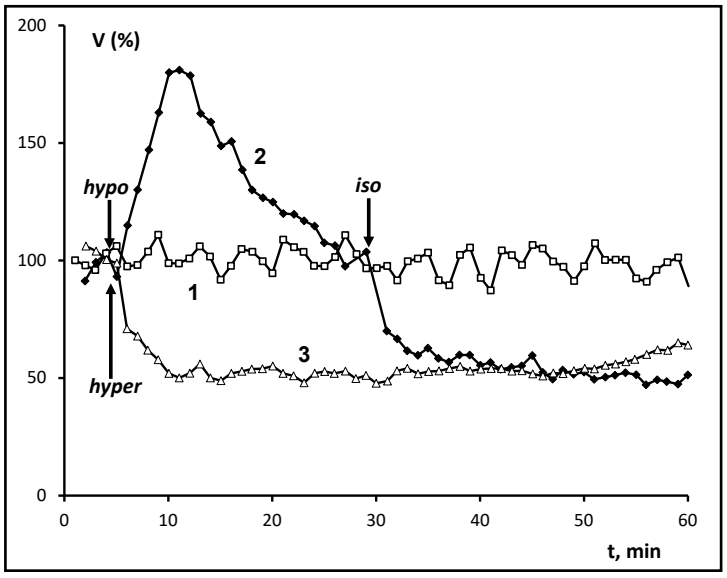

Fig. 1. Time-course of volume changes in HUVEC during 1 hr incubation in control cells (1) and in cell subjected to isosmotic (2) or hyperosmotic (3) shrinkage. Arrows indicate time when cells were exposed to hyposmotic (hypo), isosmotic (iso) and hyperosmotic (hyper) media as described in the Methods section. Initial cell volume values (V) were considered as 100%. Means for 4 independent experiments are shown.

Table 1. Effect of shrinkage and ouabain on intracellular sodium, potassium and water content in HUVEC. Cells were subjected to stimuli listed in the left column during 1 hr. Means ± S.E. obtained in 3 independent experiments are shown

Stimuli	Intracellular sodium (nmol/mg protein)	Intracellular potassium (nmol/mg protein)	Intracellular water (µl/mg protein)	$[Na^+]_i$ (mmol/litre intracellular water)	$[K^+]_i$ (mmol/litre intracellular water)
1. None (control)	123±11	779±63	7.9±0.6	15.6±1.9	98.6±11.1
2. Isosmotic shrinkage	77±16	537±53	4.3±0.2	17.9±1.3	124.8±12.9
3. Hyperosmotic shrinkage: control	143±11	501±62	4.1±0.3	34.9±4.2	122.2±16.0
4. Hyperosmotic shrinkage: Na^+-free	41±8	599±44	4.4±0.6	9.3±1.5	136.1±14.4
5. Ouabain, 0.1 µM	270±32	583±69	8.1±0.8	33.3±2.9	71.9±7.8
$p_{1,2}$	NS	NS	<0.01	NS	NS
$p_{1,3}$	NS	NS	<0.01	<0.02	NS
$p_{1,4}$	<0.001	NS	<0.02	<0.02	NS
$p_{1,5}$	<0.01	<0.001	NS	<0.01	NS

to renal epithelial cells [40, 44, 45], endothelial cells exhibit very slow RVI and in 1 hr their volume was ~60% of its value in isosmotic medium. In contrast to hyperosmotic shrinkage, swelling of HUVEC in hyposmotic medium was accompanied by rapid RVD and restoration within 10-15 min of their volume to initial values. As demonstrated in all animal cells studied so far [8, 11], transfer from hypo- to isosmotic medium resulted in sustained attenuation of HUVEC volume by 40-50%, i.e. phenomenon termed isosmotic shrinkage.

Table 1 displays that isosmotic shrinkage decreased intracellular sodium content measured by atomic absorption spectrometry (nmol/mg protein) by ~40% whereas shrinkage in hyperosmotic medium increased this parameter by ~15%. Both stimuli decreased intracellular potassium content by ~35%; these differences, however, were not statistically significant. To calculate intracellular concentration of these cations (mmol/litre), we measured the volume of intracellular water as ^{14}C-urea available space. Consistently with data obtained by DISUR technique (Fig. 1), we observed that both hyper- and isosmotic shrinkage decrease the volume of ^{14}C-urea available space by 40-50%. Keeping these values in mind we found that hyperosmotic shrinkage increased $[Na^+]_i$ by 2-fold whereas isosmotic shrinkage had no impact on this parameter (Table 1).

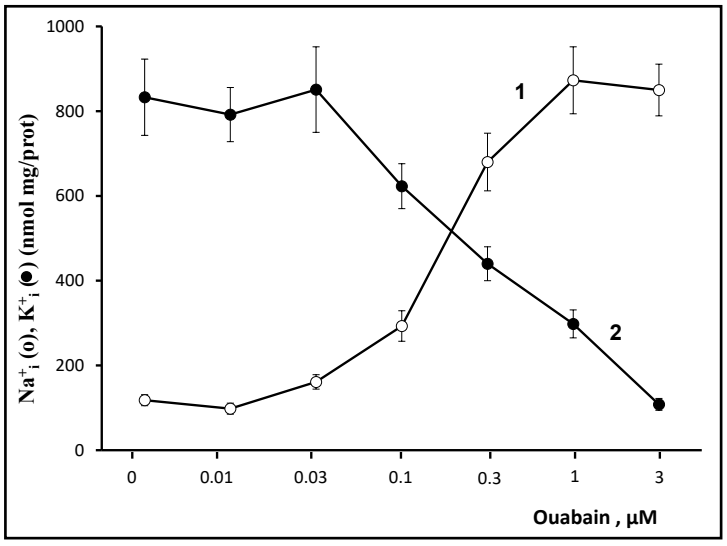

Fig. 2. Dose-dependent action of ouabain on intracellular sodium (1) and potassium (2) content in HUVEC. Cells were incubated in medium A containing ouabain for 1 hr. Means ± S.E. obtained in 3 independent experiments are shown.

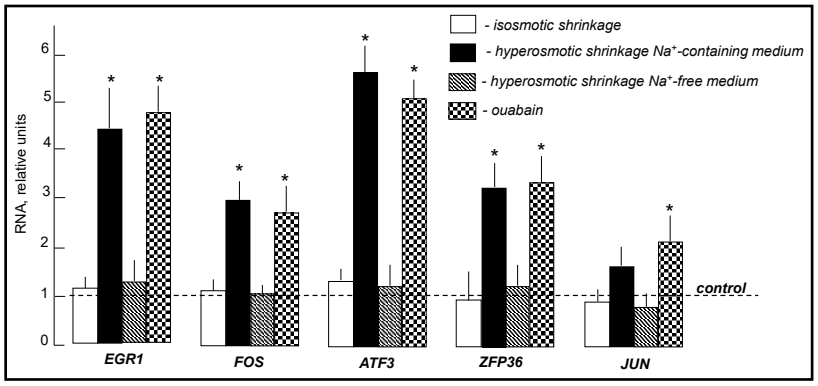

Fig. 3. Effect of cell volume modulation and ouabain on early response gene transcription in HUVEC. mRNA content in control (untreated) cells is taken as 1.00. Cells were subjected to cell volume modulation and 0.1 μM ouabain for 3 hrs. Means ± S.E. obtained in 4 independent experiments are shown. * - $p<0.05$ compared to control cells.

To examine the role of $[Na^+]_i$ elevation in transcriptomic changes evoked by cell shrinkage, we employed Na^+-free medium and ouabain as selective inhibitor of the Na^+,K^+-ATPase. Table 1 shows that substitution of NaCl by NMDG in isosmotic medium did not affect cell shrinkage but completely abolished an increment of $[Na^+]_i$ triggered by mannitol addition. Consistently with previously reported data [46], elevation of ouabain concentration from 0.03 to 3 μM resulted in dose-dependent inhibition of Na^+,K^+-ATPase indicated by the gain of intracellular Na^+ and loss of intracellular K^+ (Fig. 2). We observed that at concentration of 0.1 μM ouabain increased $[Na^+]_i$ from 15.6 to 33.3 mmol/liter intracellular water that corresponds to increment of $[Na^+]_i$ triggered by hyperosmotic shrinkage in Na^+-containing medium. Importantly, unlike hyperosmotic shrinkage addition of 0.1 mM ouabain did not affect the volume of HUVEC (Table 1).

Effect of hyper- and isosmotic shrinkage on transcription of early response genes

In previous studies we compared transcriptomic changes in several cell types including HUVEC and found dozens of common transcripts whose expression was increased up-to 20-fold under elevation of $[Na^+]_i$ triggered by sustained inhibition of Na^+,K^+-ATPase by ouabain and K^+-free medium. We noted that this set of ubiquitous Na^+_i-sensitive genes was highly abundant with ERGs and other potent transcription regulators [26, 47]. For the present study we selected EGR1, FOS, ATF3, ZFP36 and JUN, i.e. ERGs demonstrated the highest increment of mRNA content in HUVEC triggered by Na^+,K^+-ATPase inhibition [26, 47]. Keeping in mind that 1 hr exposure of HUVEC to hyperosmotic medium and 0.1 μM ouabain resulted in the

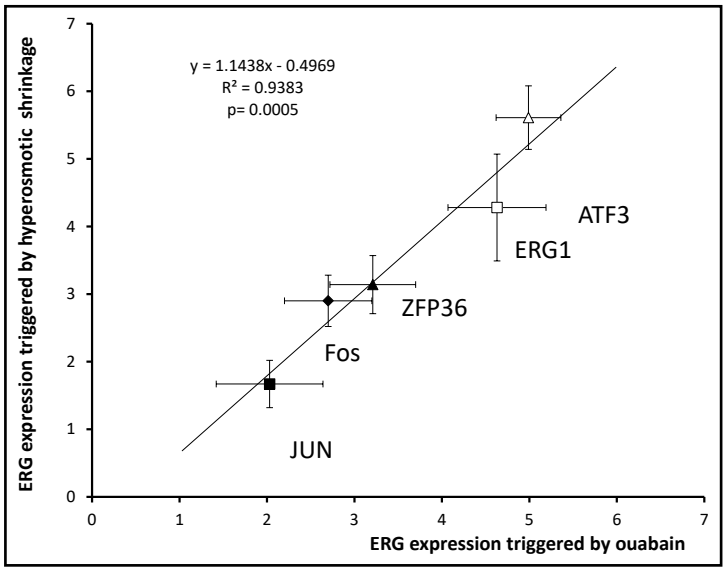

Fig. 4. Correlation analysis of early response gene expression in HUVEC triggered by 0.1 μM ouabain and hyperosmotic shrinkage in Na^+-containing medium. ERG mRNA content in control cells was taken as 1.00.

same increment of $[Na^+]_i$ (Table 1), we compared the action of these stimuli on the content of mRNAs encoding Na^+_i-sensitive ERGs detected in previous studies.

Both, exposure of HUVEC to 0.1 μM ouabain and hyperosmotic shrinkage in Na^+-containing medium increased the content of mRNAs encoding EGR1, FOS, ATF3, ZFP36 and JUN by ~4, 3, 5, 3 and 2-fold, respectively (Fig. 3). Importantly, we observed highly significant (p=0.0005) positive (R^2=0.9383) correlation between levels of differentially expressed transcripts identified in shrunken cells and in the presence of ouabain (Fig. 4). Substitution of Na^+ by NMDG completely abolished the effect of hyperosmotic shrinkage on gene transcription. Unlike hyperosmotic shrinkage we failed to detect any significant action of isosmotic shrinkage on the transcription of 5 ERGs been under investigation (Fig. 3).

Discussion

An implication of NFAT5 in augmented expression of genes involved in accumulation of organic osmolytes and delayed RVI is well-documented [17-19]. It should be noted, however, that NFAT5-mediated pathways can not be considered as a universal mechanism of transcription regulation in hyperosmotically shrunken cells. Indeed, unlike SMIT, BGT1 and TauT augmented content of NTE mRNA in shrunken cells is mediated by NFAT5-independent signaling [18]. It was shown that elevation of intracellular glycerol content via expression of glycerol-3-phosphate dehydrogenase (*Gpdg*) is the only mechanism of *C. elegans* protection in hypertonic environment. Nor TonEBP, neither its structural analogue contributes to this phenomenon [48]. Numerous research teams demonstrated that side-by-side with cell shrinkage NFAT5-mediated mechanism of transcription regulation might be caused by diverse signaling pathways triggered by nitric oxide, angiotensin II, TNF-α, TGF-β including activation of Ca^{2+}-mediated and -independent isoforms or protein kinase C (for review, see [49]).

Previously, it was shown that elevation of $[Na^+]_i$ results in rapid accumulation of RNAs encoding FOS, JUN and other ERGs [26, 50, 51]. Our results strongly suggest that elevation of $[Na^+]_i$ plays a key role in augmented transcription of these genes in hyperosmotically shrunken cells. This conclusion is supported by evidences listed below.

First, hyperosmotic shrinkage of HUVEC resulted in 2-fold elevation of $[Na^+]_i$ and elevation of ZFP36, EGR1, FOS, ATF3 and JUN mRNAs (Table 1, Fig. 1 and 3). Importantly, 5-fold elevation of ZFP36 was observed in 3 hr of exposure of HUVEC to hyperosmotic

medium (Fig. 3) contrasting with delayed expression of NFAT5-dependent osmoprotecive genes documented in 24-48 hrs [52].

Second, partial inhibition of the Na^+,K^+-ATPase in presence of 0.1 μM ouabain led to the same elevation of $[Na^+]_i$ as detected in hyperosmotically shrunken cells (Table 1). At this concentration, actions of ouabain and hyperosmotic medium on ERG transcription exhibited highly significant positive correlation (Fig. 4).

Third, neither $[Na^+]_i$ nor the content of ERG mRNAs was affected by shrinkage triggered by transfer of HUVEC from hypo- to isosmotic medium (Table 1, Fig. 3).

Forth, the isosmotic substation of Na^+ by NMDG completely abolished the increment of $[Na^+]_i$ triggered by mannitol addition without any significant impact on the volume of intracellular water (Table 1). These results show that the gain of $[Na^+]_i$ rather than attenuation of cell volume *per se* is responsible for transcription activation. Importantly, NFAT5-mediated transcription of osmoprotective SMIT, BGT1, TauT and AR was observed when medium osmolality was increased by NaCl or membrane-impermeable mannitol whereas urea and other membrane-permeable osmolytes did not affect this signaling system [53]. These observations strongly suggest that NFAT5-mediated signaling is triggered by elevated ionic strength rather than by selective increment of $[Na^+]_i$.

Are endothelial cells exposed to hypertonic environment in the kidney microcirculation and during local inflammation? Do Na^+-sensitive early response genes contribute to protection of vascular endothelium in hyperosmotic conditions? Do hypertonicity-induced cation channels (HICC) play a key role in $[Na^+]_i$ elevation detected in hyperosmotically shrunken HUVEC? What is the molecular origin of upstream $[Na^+]_i$ sensor involved in activation of ERG transcription? We address these questions in the forthcoming studies.

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Disclosure Statement

The authors declare no competing financial interests.

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