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# Vascular Smooth Muscle as an Oxygen Sensor: Role of Elevation of the $[Na^+]_i/[K^+]_i$

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## Abstract

The article presents a review of data from our own research and data obtained by other authors about the role of intracellular sodium ( $Na_i^+$ ) and potassium ( $K_i^+$ ) in transcriptional changes in vascular smooth muscle cells (VSMC) during hypoxia. It was found that acute hypoxia suppressed  $[K^+]_o$  and phenylephrine-induced contractions of aortic rings through voltage-gated as well as by  $Ca_i^{2+}$ - and ATP-sensitive  $K^+$  channels; 24-h incubation of VSMC in ischemic conditions resulted in attenuation of ATP content, elevation of  $[Na^+]_i$  and loss of  $[K^+]_i$ . Dissipation of  $Na^+$  and  $K^+$  gradients in low- $Na^+$ , high- $K^+$  medium completely eliminated increment in Fos, Atf3, Ptgs2 and Per2 mRNAs and sharply diminished augmentation of Klf10, Edn1, Nr4a1 and Hes1 expression evoked by hypoxia. All these data suggest that  $Na_i^+/K_i^+$ -mediated signaling contribute to transcriptional changes in VSMC subjected to sustained hypoxia.

**Keywords:** smooth muscle cells, hypoxia, intracellular  $[Na^+]/[K^+]$  ratio, transcription, contraction

## 1. Introduction

Maintaining optimal oxygen tension level in cells promotes the metabolic and plastic processes that ensure their functional stability. To date, there are a lot of reports showing the high sensitivity of endothelium-denuded blood vessels to oxygen deficiency (hypoxia) [1–5]. These data allow considering vascular smooth muscle cells (VSMC) as an oxygen sensor involved in modulation of blood vessel tone and gene expression. Previously, using global gene expression profiling, we found that in several cell types including rat aortic VSMC  $Na^+$ ,  $K^+$ -ATPase inhibition by

ouabain or  $K^+$ -free medium led to the differential expression of dozens of genes whose altered expression was previously detected in cells subjected to hypoxia and ischemia/reperfusion [6, 7]. In view of this finding, we examined the relative impact of canonical hypoxia-inducible factor 1alpha (HIF-1 $\alpha$ )- and  $Na_i^+/K_i^+$ -mediated signaling on transcriptomic changes evoked by hypoxia and glucose deprivation as well as its possible involvement in regulation of VSMC contraction.

## 2. Hypoxia affects excitation-contraction and excitation-transcription coupling: role of HIF-1 $\alpha$ -mediated signaling

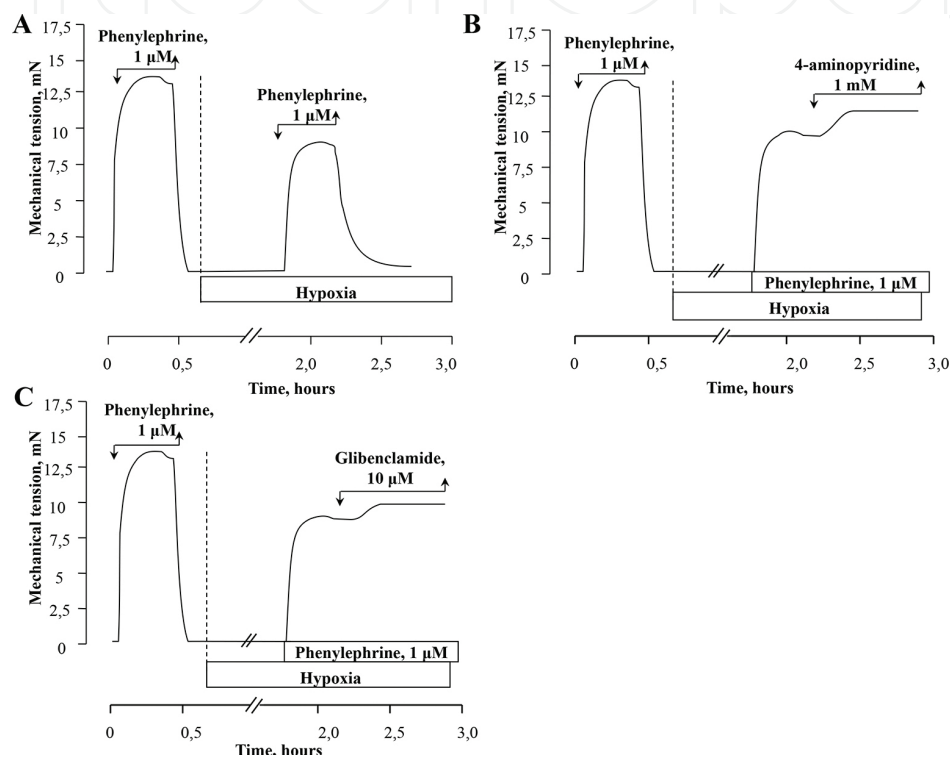
Blood vessels play a key role in the maintenance of a balanced supply of oxygen and nutrition in target tissues under acute and chronic hypoxic conditions. In systemic circulation, acute hypoxic conditions resulted in dilatation of vascular beds via direct actions of attenuated partial oxygen pressure ( $pO_2$ ) on vascular smooth muscle cells (VSMC) as well as by ATP release from erythrocytes that, in turn, leads to activation of purinergic P2Y receptors and augmented production of nitric oxide by endothelial cells (for comprehensive reviews, see [1–3]).

**Figure 1A** shows that in the absence of erythrocytes, hypoxia attenuated by 20–30% the contraction of rat aortic strips triggered by agonist of  $\alpha_1$ -adrenergic receptors phenylephrine. We found that inhibitory action of hypoxia was partially abolished by 4-aminopyridine (**Figure 1B**) and glibenclamide (**Figure 1C**), thus indicating activation of voltage-gated and ATP-sensitive  $K^+$  channels, respectively. Recently, Gun et al. reported that hypoxic relaxation of mesenteric arteries is suppressed by a selective inhibitor of the large conductance  $Ca^{2+}$ -activated  $K^+$  channels ( $BK_{Ca}$ ) iberiotoxin [4].

Unlike systemic circulation, hypoxia results in augmented contraction of pulmonary arterial smooth muscle cells via inhibition of voltage-gated  $K^+$  channels  $K_v1.5$  and  $K_v2.1$  and activation of nonselective cation channels TRPC1 (for reviews, see [5, 8]). It was shown that ATP release from erythrocytes triggered by shear stress and activation of cAMP-mediated signaling is sharply decreased in human with primary pulmonary hypertension [9]. To the best of our knowledge, the comparative analysis of hypoxia-induced ATP release from erythrocytes of normotensive and hypertensive patients and implication of purinergic receptors in regulation of vascular tone in systemic and pulmonary circulations have not yet been performed.

In addition, the regulation of vascular tone hypoxia leads to cell type-specific differential expression of hundreds of genes documented in global gene profiling studies [10–16]. It is generally accepted that these transcriptomic changes are mediated by hypoxia-inducible factor 1alpha (HIF-1 $\alpha$ ) involved in regulation of gene expression via interaction of HIF-1 $\alpha$ /HIF-1 $\beta$  heterodimer with hypoxia-response elements (HREs) in promoter/enhancer regions of the target gene's DNA. In normoxia, oxygen-dependent prolyl hydroxylase hydroxylates HIF-1 $\alpha$  and induces its proteasomal degradation. In contrast, under hypoxic conditions, HIF-1 $\alpha$  is translocated to the nucleus, where it forms HIF-1 $\alpha$ /HIF-1 $\beta$  complex [17–20]. The list of HIF-1-sensitive genes includes Hif-1 $\alpha$  per se and others related to angiogenesis (vascular endothelial growth factor (Vegf) and its receptor Flt1), vasomotor control (endothelin-1, adrenomedullin,

nitric oxide synthase-2), erythropoiesis and iron metabolism (transferrin, transferrin receptor, erythropoietin, ceruloplasmin), energy metabolism (phosphoenolpyruvate carboxylase, aldose, endolase, phosphoglucokinase-1, -L and -C, lactate dehydrogenase A, tyrosine hydroxylase and plasminogen activator inhibitor-1, glucose transporters Glut1-Glut3), and cell proliferation (Tgfb, Igf1, Igfbp1) [21]. Shimoda and coworkers reported that reduction in voltage-gated  $K^+$  currents following hypoxia was absent in pulmonary arterial smooth muscle cells from heterozygous HIF-1 $\alpha$  mice, thus suggesting and implicating this oxygen-sensing machinery in vascular bed-specific contractile responses [22].



**Figure 1.** Hypoxia influences on phenylephrine (PE)-induced contraction of ring aortic segments from male Wistar rats. Aortic segments were incubated for 60 min in hypoxic Krebs solution ( $pO_2 \sim 30$  mmHg) and then contacted with phenylephrine (1  $\mu$ M). Registration of constrictive responses was performed by Myobath-2 Multi-Channel Tissue Bath System. Incubation in hypoxic solution decreased the amplitude of PE-induced constriction in comparison with contraction in normoxic solution (A). Both blocker of voltage-dependent potassium channel 4-aminopyridine (1 mM) (B) and blocker of ATP-dependent potassium channels glibenclamide (10  $\mu$ M) (C) significantly decreased mechanical tension of aortic segments in comparison with PE-induced contraction in hypoxic solution ( $p < 0.05$ ). X axis—time (h), Y axis—mechanical tension (mN). The arrows indicate the addition and removal of the respective solutions.

It should be noted that side-by-side with activation of HIF-1 $\alpha$ -mediated signaling, attenuation of  $pO_2$  and delivery of cell fuels resulted in decreased intracellular ATP content that, in turn, led to activation of AMP-sensitive protein kinase (AMPK) [23, 24], decline of ion transport ATPase activities and dissipation of electrochemical gradients of  $K^+$ ,  $Na^+$ ,  $Cl^-$  and  $Ca^{2+}$  [25]. Numerous research teams reported that  $[Ca^{2+}]_i$  elevation triggers transcriptomic alterations via  $Ca_i^{2+}$ -sensitive transcriptional elements [26]. Importantly, along with the increment in  $[Ca^{2+}]_i$ , even transient ischemia increases  $[Na^+]_i$  from 5–8 to 25–40 mM and causes reciprocal changes

in  $[K^+]_i$  [27]. These data motivate us to propose that  $Na^+_i/K^+_i$ -sensitive signaling pathways contribute to cellular responses triggered by sustained hypoxia [6, 28]. Investigations examining this hypothesis are considered below.

### 3. Intracellular monovalent cations as regulators of gene transcription

In the late 1990s, we observed that elevation of the  $[Na^+]_i/[K^+]_i$  ratio protects rat aortic VSMC against apoptosis triggered by serum deprivation and staurosporine addition [29]. To further explore this antiapoptotic pathway, we treated cells with actinomycin D or cycloheximide. Both macromolecular synthesis inhibitors abolished protection against apoptosis by ouabain [30]. Later we employed proteomic technology and detected hundreds of differentially expressed protein spots in VSMC subjected to  $Na^+$ ,  $K^+$ -ATPase inhibition by ouabain and other cardiotonic steroids (CTS) [30]. These data, together with augmented RNA synthesis observed in ouabain-treated VSMC [31], suggest that sharp transcriptomic changes seen in ouabain-treated cells are mediated by immediate response genes (IRG). Indeed, in both RASMC and HeLa cells, ouabain treatment resulted in augmentation of immunoreactive c-Fos and c-Jun by 10-fold and fourfold, respectively [32, 33]. Addition of ouabain induced a fourfold c-Fos mRNA increment accompanied by fivefold increment in  $[Na^+]_i$  within 30 min. At the same time, we observed only 10–15% decrease in  $[K^+]_i$  [32, 33]. Thus, we can assume that c-Fos expression is more sensitive to increase in  $[Na^+]_i$  rather than  $[K^+]_i$ .

Recent studies have revealed that CTS may affect cells independently of suppression of  $Na^+$ ,  $K^+$ -ATPase. Thus, ouabain induced interaction of  $\alpha$ -subunit of the  $Na^+$ ,  $K^+$ -ATPase with the membrane-associated nonreceptor tyrosine kinase Src, activation of Ras/Raf/ERK1,2, phosphatidylinositol 3-kinase (PI(3)K), PI(3)K-dependent protein kinase B, phospholipase C,  $[Ca^{2+}]_i$  oscillations and increased production of the reactive oxygen species (for review, see [34–36]). Considering this, we employed  $K^+$ -free medium as an alternative approach for  $Na^+$ ,  $K^+$ -ATPase inhibition. To identify  $Na^+_i/K^+_i$ -sensitive transcriptomes, both ubiquitous and cell type-specific, we compared the effect of ouabain and  $K^+$ -free medium on profiles of gene expression in rat VSMC, human umbilical vein endothelial cells (HUVEC) and the human carcinoma HeLa cell line [26]. Using Affymetrix-based technology, we found that expression of 684, 737 and 1839 transcripts in HeLa, HUVEC and RASMC, respectively, changes up to 60-fold. It is worth noting that there was a strong correlation in cells pretreated with ouabain or  $K^+$ -free medium for 3 h. We also found that 80 transcripts of examined  $Na^+_i/K^+_i$ -sensitive genes were common for all examined types of cells [26].

We found that genes involved in the regulation of transcription represents a half of ubiquitous  $Na^+_i/K^+_i$ -sensitive transcriptome. This amount was ~sevenfold higher than in the total human genome [37]. The group of ubiquitous  $Na^+_i/K^+_i$ -sensitive genes, whose expression was increased by more than threefold, included the transcription factor of the steroid-thyroid

hormone-retinoid receptor superfamily Nr4a2, transcriptional regulator of C2H2-type zinc finger protein Egr-1, the basic helix-loop-helix transcription regulator Hes1, members of the superfamily of b-zip transcriptional factors possessing leucine-zipper dimerization motif and basic DNA-binding domain and forming heterodimeric activating protein AP-1 (Fos, FosB, Jun, JunB, Atf3) [26].

#### 4. Evidence for $Na_i^+/K_i^+$ -mediated, $Ca_i^{2+}$ -independent excitation-transcription coupling

Because of the high electrochemical gradient, the opening of calcium channels resulted in rapid elevation of  $[Ca^{2+}]_i$  from  $\sim 0.1$  to  $1 \mu M$ , its interaction with calmodulin and other  $[Ca^{2+}]_i$  sensors, in turn, affects the expression of hundreds of genes, i.e., phenomenon termed excitation-transcription coupling [38]. Increase in  $[Ca^{2+}]_i$  affects transcription via several signaling pathways. Thus,  $[Ca^{2+}]_i$  elevation induces translocation of kappa-light-chain enhancer of nuclear factor (NF $\kappa$ B) of activated B cells from the cytosol to the nucleus. This process is triggered by activation of  $Ca^{2+}$ /calmodulin-sensitive protein kinase (CaMKI, II or III) and phosphorylated I $\kappa$ B kinase that phosphorylates the inhibitor of  $\kappa$ B (I $\kappa$ B) [38].  $[Ca^{2+}]_i$  elevation also promotes translocation from cytosol to the nucleus; nuclear factor of activated T cells (NFAT) is evoked by its dephosphorylation by the ( $Ca^{2+}$ /calmodulin)-dependent phosphatase calcineurin [39]. In addition, increased cytosolic and nucleoplasmic  $Ca^{2+}$  concentrations lead to phosphorylation of cAMP response element-binding protein (CREB) by CaMKII and CaMKIV, respectively. Phosphorylated CREB regulates transcription via their binding to the ( $Ca^{2+}$ +cAMP)-response element (CRE) sequences of DNA [40].

Because the c-Fos promoter contains CRE, its augmented expression might be mediated by depolarization of ouabain-treated VSMC and the opening of voltage-gated  $Ca^{2+}$  channels. However, unlike high- $K^+$  medium, c-Fos expression in ouabain-treated cells was not affected by inhibition of L-type  $Ca^{2+}$  channels with nifedipine [41]. In additional experiments, we found that augmented c-Fos expression evoked by ouabain was preserved in  $Ca^{2+}$ -free medium and in the presence of extracellular (EGTA) and intracellular (BAPTA)  $Ca^{2+}$  chelators [30]. To study the role of  $Ca_i^{2+}$ -mediated and  $Na_i^+/K_i^+$ -independent signaling, we compared transcriptional changes triggered by elevation of the  $[Na^+]_i/[K^+]_i$  ratio in control and  $Ca^{2+}$ -depleted cells. Depletion of  $Ca^{2+}$  led to prevalent increase in  $Na_i^+/K_i^+$ -sensitive genes, both ubiquitous and cell-type specific [26]. For further investigation, we examined ubiquitous  $Ca_i^{2+}$ -sensitive genes whose expression is regulated by more than threefold independently of the presence of  $Ca^{2+}$  chelators and selected several transcription factors (Fos, Hes1, Nfkb1a, Jun), protein phosphatase 1, dual specificity phosphatase Dusp8, interleukin-6, regulatory subunit, type 2 cyclooxygenase COX-2, cyclin L1 [41].

Considering these data, it is important to underline that  $Ca^{2+}$  chelators may affect cellular functions independently of  $Ca^{2+}$  depletion. Thus, we observed that the addition of EGTA

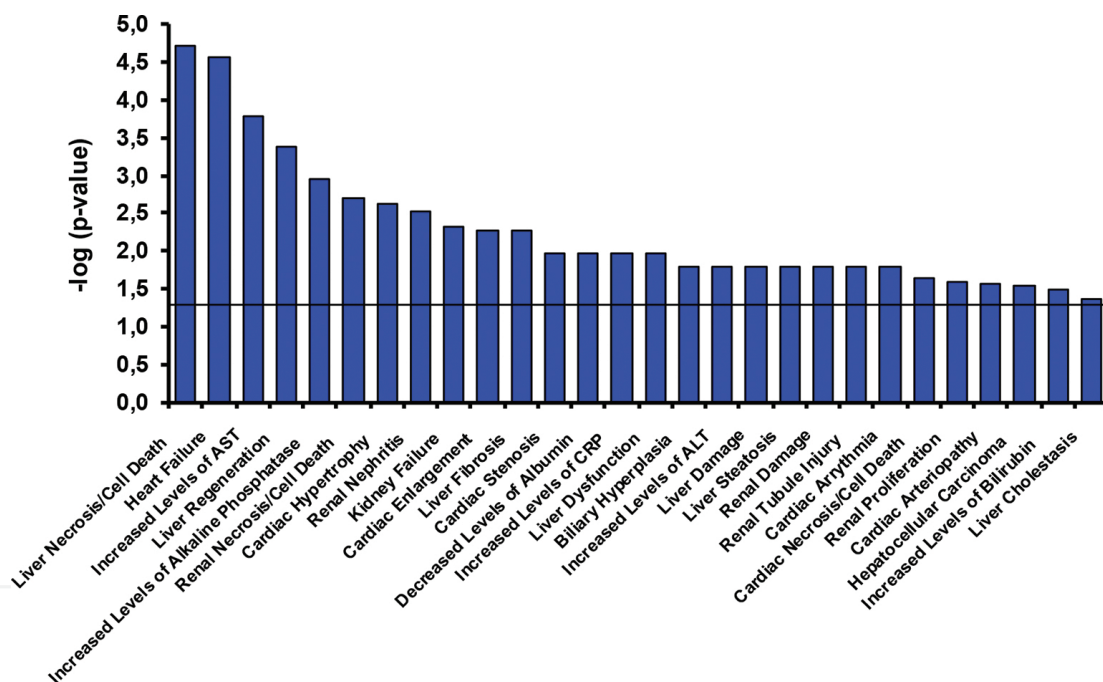
increases permeability of VSMC for  $\text{Na}^+$  [41]. It is also known that the affinity of EGTA for  $\text{Mn}^{2+}$ ,  $\text{Zn}^{2+}$ ,  $\text{Cu}^{2+}$ ,  $\text{Co}^{2+}$ ,  $\text{Fe}^{2+/3+}$  is 10-fold to  $10^7$ -fold higher than for  $\text{Ca}^{2+}$  [42–44]. These polyvalent cations are important in regulation of metalloenzymes activity and participate in protein-DNA and protein-protein interactions. Moreover, EGTA causes irreversible conformational transition and inactivation of transcriptional adaptor  $\text{Zn}^{2+}$ -binding domain that affects gene expression [45]. It is worth noting that in the human genome, the C2H2 zinc finger superfamily includes about half of all annotated transcription factors [46]. This implies that this and other chelators have  $\text{Ca}_i^{2+}$ -independent action on transcriptomic changes evoked by diverse stimuli. Keeping these data in mind, we compared the actions of  $\text{Ca}^{2+}$  chelators and  $\text{Na}^+$ ,  $\text{K}^+$ -ATPase inhibitors on transcriptomic changes and concentration of monovalent cations in VSMC [47]. Our results show that transcriptomic changes seen in  $\text{Ca}^{2+}$ -depleted VSMC are at least partially caused by elevation of the  $[\text{Na}^+]_i/[\text{K}^+]_i$  ratio and activation of  $\text{Na}_i^+/\text{K}_i^+$ -independent signaling pathways. This conclusion is supported by several observations. First,  $\text{Ca}^{2+}$  depletion led to a ~threefold elevation of  $[\text{Na}^+]_i$  and a twofold attenuation of  $[\text{K}^+]_i$ . An increment in the  $[\text{Na}^+]_i/[\text{K}^+]_i$  ratio seen in  $\text{Ca}^{2+}$ -depleted cells was caused by elevation of plasma membrane permeability for monovalent cations. Indeed,  $\text{Ca}^{2+}$  depletion resulted in almost threefold elevation of the rate of  $^{22}\text{Na}$  and  $^{86}\text{Rb}$  influx measured in the presence of inhibitors of  $\text{Na}^+$ ,  $\text{K}^+$ -ATPase and  $\text{Na}^+$ ,  $\text{K}^+$ ,  $2\text{Cl}^-$  cotransport. Second, the list of genes whose mRNA content was increased in  $\text{Ca}^{2+}$ -depleted cells by more than fourfold includes a large number of genes whose expression was also attenuated by the  $\text{Na}^+$ ,  $\text{K}^+$ -ATPase inhibition in  $\text{K}^+$ -free medium. Third, there was a strong positive correlation in mRNA content of 2071 genes whose expression was changed by more than 1.2-fold in cells subjected to  $\text{Na}^+$ ,  $\text{K}^+$ -ATPase inhibition in  $\text{K}^+$ -free medium as well as in  $\text{Ca}^{2+}$ -depleted cells. Fourth, dissipation of transmembrane gradients of  $\text{Na}^+$  and  $\text{K}^+$  in high- $\text{K}^+$ , low- $\text{Na}^+$  medium abolished the increment in the  $[\text{Na}^+]_i/[\text{K}^+]_i$  ratio as well as sharp elevation of *Atf3*, *Nr4a1* and *Erg3* mRNA content triggered by 3-h incubation of VSMC in  $\text{Ca}^{2+}$ -free, EGTA-containing medium [47]. Thus, novel molecular biological and pharmacological approaches should be developed for precise identification of the relative impact of  $\text{Ca}^{2+}$ -mediated and  $\text{Ca}^{2+}$ -independent pathways on transcriptomic changes evoked by elevation of the  $[\text{Na}^+]_i/[\text{K}^+]_i$  ratio.

## 5. Evidence for implication of $[\text{Na}^+]_i/[\text{K}^+]_i$ -sensitive pathways in transcriptomic changes evoked by hypoxia

The crosstalk between transcriptomic changes and monovalent ion handling was initially supported by comparative analysis of  $\text{Na}_i^+/\text{K}_i^+$ -sensitive genes documented in our investigations [26] and data on genes whose expression in hypoxic conditions was changed in studies performed by other research groups [9, 11, 48–56]. Indeed, among genes whose augmented expression was detected both in vivo and in vitro models of ischemia/reperfusion, we found several ubiquitous  $\text{Na}_i^+/\text{K}_i^+$ -sensitive genes, including transcription factors *EGR1*, *ATF3*, *NFKBIZ*, *HES1* as well as type 2 cyclooxygenase, *IL6*, thioredoxin-interacting protein *TXNIP*.

Moreover, using IPA-knowledge base data, we observed that ubiquitous  $Na^+_i, K^+_i$ -sensitive transcriptomes are highly significantly correlated with differential expression of genes in disorders triggered by kidney, liver and heart ischemia (**Figure 2**). These data allowed us to propose that transcriptomic changes in ischemic tissues are at least partially mediated by a novel  $Na^+_i, K^+_i$ -mediated excitation-transcription coupling [26, 27].

To examine this hypothesis, we compared the effect of ouabain and hypoxia on the content of monovalent ions and ATP in VSMC from the rat aorta. We observed that 24-h incubation of VSMC in hypoxia and glucose starvation decreased intracellular ATP content by ~three-fold, whereas ouabain attenuated this parameter by <20% (**Figure 3**). Ouabain led to almost 10-fold increase in  $[Na^+]_i$  and similar decrease in  $[K^+]_i$ . Hypoxia also caused threefold increase in  $[Na^+]_i$  and twofold decrease in  $[K^+]_i$ . At the same time, reduction in monovalent cations transmembrane gradients in low- $Na^+$ , high- $K^+$  medium almost completely eliminated the actions of ouabain and hypoxia on the  $[Na^+]_i/[K^+]_i$  ratio [57].

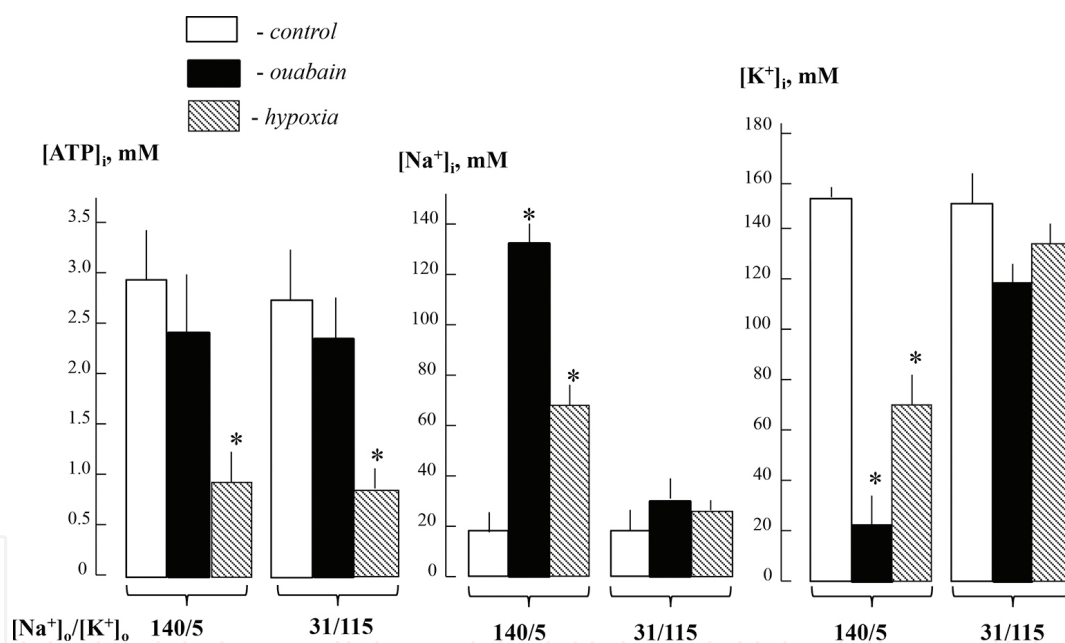


**Figure 2.** Disorders significantly associated with differential expression of genes whose expression was ubiquitously changed in VSMC from rat aorta, human umbilical vein endothelial cells and HeLa cell line subjected to  $Na^+, K^+$ -ATPase inhibition by both ouabain and  $K^+$ -free medium. The criteria with a threshold for significance of  $p = 0.05$  (or 1.3 when expressed as  $-\log(p\text{-value})$ ) are shown as straight line. Adopted with permission from [26].

We then identified the  $[Na^+]_i/[K^+]_i$ -sensitive transcriptome in rat VSMC. We found that 6-h inhibition of the  $Na^+, K^+$ -ATPase with ouabain or in  $K^+$ -free medium resulted in differential expression of 6412 transcripts exhibit highly significant ( $p < 4 \times 10^{-9}$ ) and positive ( $R^2 > 0.80$ ) correlation and classified as  $Ca^{2+}_i$ -sensitive genes [57]. To continue our studies, we selected genes whose participation in the pathogenesis of hypoxia was shown in previous studies combined with the property of the highest expression increments under sustained  $Na^+, K^+$ -



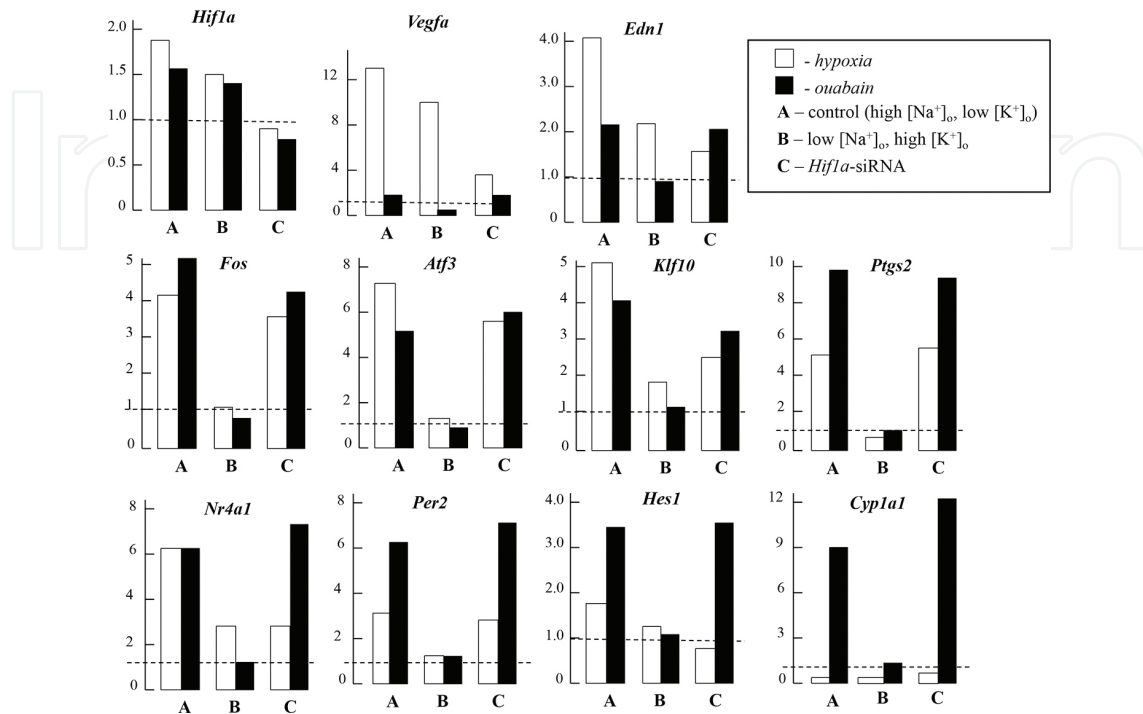
ATPase inhibition. These genes include Fos, Cyp1a1, Klf10, Atf3, Nr4a1, Hes1, Ptgs2 and Per2. Among these genes, Fos, Atf3 and JUN together form dimeric transcription factor AP-1 whose expression increased in all types of cells subjected to hypoxia [58]. Klf10 is a Kruppel-like zinc finger transcription factor family member involved in hypoxia-dependent angiogenesis via COX-1 activation [59]. Ptgs2 encodes an inducible isoform of cyclooxygenase-2 (COX-2) whose role in the pathophysiology of hypoxia is well documented [60]. Nur77 or Nr4a1, also known as nerve growth factor IB, is the nuclear receptor of transcription factors stabilizing HIF-1 $\alpha$  which increases its transcriptional activity [61]. Hes1 is the main helix-loop-helix transcription factor that enhances the expression after ischemic renal failure [52]. Clock, Bmal1, Per1, Per2, Cry1 and Cry2 are the positive (Clock and Bmal1) and negative (others) regulators of a transcription-translation feedback loop forming the core circadian oscillator [62]. Cyp1a1 encodes a cytochrome P450 family member and its expression is mediated by HIF-1 $\beta$  [63, 64]. Per2 promotes circadian stabilization of HIF-1 $\alpha$  activity that is critical for myocardial adaptation to ischemia. The positive controls for canonical HIF-1 $\alpha$ -sensitive genes are endothelin (Edn1) and vascular endothelial growth factor (Vegfa).



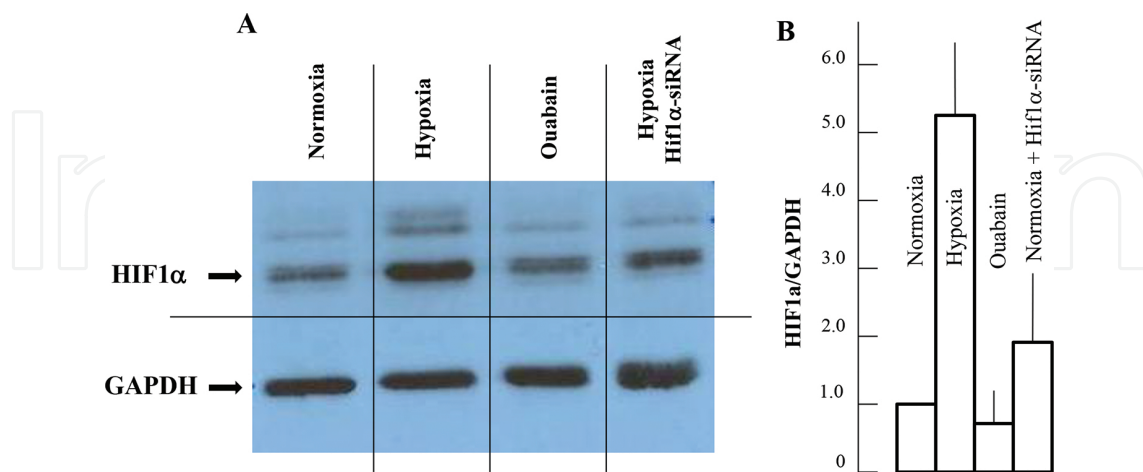
**Figure 3.** Effect of ouabain and hypoxia on intracellular Na<sup>+</sup>, K<sup>+</sup> and ATP concentrations in VSMC from the rat aorta. Cells were exposed to normal oxygen partial pressure (5% CO<sub>2</sub>/air—control) ±3 μM ouabain or exposure to hypoxia (5% CO<sub>2</sub>/95% N<sub>2</sub>)/glucose deprivation for 24 h in normal high-Na<sup>+</sup>, low-K<sup>+</sup> ([Na<sup>+</sup>]/[K<sup>+</sup>] = 140/5) or in low-Na<sup>+</sup>, high-K<sup>+</sup> DMEM-like medium ([Na<sup>+</sup>]/[K<sup>+</sup>] = 131/115). Intracellular K<sup>+</sup> and Na<sup>+</sup> Cl<sup>-</sup> content was measured as the steady-state distribution of extra- and intracellular <sup>86</sup>Rb and <sup>22</sup>Na, respectively. Intracellular ATP content was measured by assaying luciferase-dependent luminescence with ATP bioluminescent assay kit. Means ± S.E. from three independent experiments performed in quadruplicate are shown. \**p* < 0.05 compared to the controls. Adopted with permission from [57].

To assess the role of [Na<sup>+</sup>]<sub>i</sub>/[K<sup>+</sup>]<sub>i</sub>-dependent and HIF-1 $\alpha$ -mediated signaling, we compared expression of the above-listed selected genes in hypoxic conditions and under the action of ouabain in control high-Na<sup>+</sup>, low-K<sup>+</sup> medium and in high-K<sup>+</sup>, low-Na<sup>+</sup> medium with dissipated transmembrane gradients of monovalent cations and after cells transfection with Hif-1a siRNA

[57]. As demonstrated in other cell types [65, 66], hypoxia slightly enhanced Hif-1 $\alpha$  mRNA (Figure 4) but increased immunoreactive HIF-1 $\alpha$  protein content by ~fivefold (Figure 5).



**Figure 4.** Effect of hypoxia and ouabain on gene expression in VSMC from the rat aorta. Cells were incubated for 24 h under normoxia, hypoxia/glucose deprivation or 3 mM ouabain in control high- $Na^+$ , low- $K^+$  medium (A, C), or high- $K^+$ , low- $Na^+$  medium (B). In some experiments, RASMC were transfected with Hif-1 $\alpha$  siRNA (C). The content of mRNA in normoxia was taken as 1.00 and shown as broken lines. Adopted with permission from [57].



**Figure 5.** (A). Representative Western blots of GAPDH and HIF-1 $\alpha$  in VSMC incubated for 24 h under control conditions (normoxia), hypoxia/glucose deprivation, 3 mM ouabain or hypoxia/glucose deprivation in cells transfected with Hif-1 $\alpha$  siRNA. (B). Hypoxia/glucose deprivation and ouabain influence on HIF-1 $\alpha$  protein relative content in RASMC. The HIF-1 $\alpha$ /GAPDH ratio in control conditions was taken as 1.00. Data obtained in three independent experiments are reported as means  $\pm$  S.E. Adopted with permission from [57].

Transfection of rat VSMC with Hif-1 $\alpha$  siRNA but not with scrambled siRNA led to ~threefold expression reduction in Hif-1 $\alpha$  and lowered hypoxia-induced HIF-1 $\alpha$  protein gain (**Figure 5**). Pretreatment with ouabain slightly changed HIF-1 $\alpha$  protein content (**Figure 5**) and amplified baseline Hif-1 $\alpha$  mRNA by ~50% (**Figure 4**). Hypoxia causes fourfold and 12-fold increase in Edn1 and Vegfa mRNA content, respectively, (**Figure 4**), which is consistent with earlier observations [19]. Hypoxia-dependent increase in Edn1 and Vegfa mRNA was attenuated after transfection with Hif-1 $\alpha$  siRNA by ~twofold and fourfold, respectively. At the same time, ouabain augmented Edn1 mRNA by 2.5-fold but did not significantly impair Vegfa. Similarly, low-Na<sup>+</sup>, high-K<sup>+</sup> medium that is characterized with dissipation of the transmembrane gradients of monovalent cations also did not affect hypoxia-induced expression of Vegfa and reduced Edn1 mRNA by twofold. All these data strongly support the efficacy of Hif1 $\alpha$ -siRNA function [57].

In hypoxic conditions, dissipation of monovalent cations transmembrane gradients completely suppressed increments in Fos, Atf3, Ptgs2 and Per2 mRNA and diminished increase in Klf10, Edn1, Nr4a1 and Hes1 expression (**Figure 4**). Hypoxia caused from twofold to sixfold augmentation of Atf3, Fos, Ptgs2, Klf10, Nr4a2, Hes1 and Per2 expression (**Figure 4**). These data are consistent with the observations obtained in other cell types, including human VSMC [67, 68]. Transfection with Hif-1 $\alpha$  siRNA led to twofold attenuation of hypoxia-induced increase in Nr4a and Klf10 mRNA without significant influence on expression of Fos, Atf3, Ptgs2 and Per2 evoked by hypoxia. At the same time, hypoxic conditions led to twofold decrease in Cyp1a1 mRNA and attenuated expression of Cyp1a1 obtained from human microvasculature [69]. Ouabain enhanced the expression of all eight tested genes from threefold to 10-fold that were completely abolished in low-Na<sup>+</sup>, high-K<sup>+</sup> medium characterized with dissipation of the transmembrane gradients of monovalent cations [57]. However, in ouabain-treated RASMC, the expression of these genes was not affected by transfection with Hif-1 $\alpha$  siRNA, but decrease in monovalent cations transmembrane gradient sharply decreased elevation of Edn1, Klf10, Hes1 and Nr4a1 expression seen in hypoxic conditions and completely abolished increase in Atf3, Fos, Ptgs2 and Per2 mRNA (**Figure 4**).

## 6. Unresolved issues and future directions

Viewed collectively, our results demonstrate a key role of [Na<sup>+</sup>]<sub>i</sub>/[K<sup>+</sup>]<sub>i</sub>-mediated excitation-transcription coupling in overall transcriptomic changes triggered by sustained ischemia. The molecular organization of sensors for monovalent cation is still unclear in contrast to rapid progress in the identification of Ca<sub>i</sub><sup>2+</sup> sensors. Initially, such sensors were identified in parvalbumin and calmodulin. These high-affinity binding sites (the so-called EF-hand domains) are formed by a highly conservative linear amino acid sequence consisting of 14 amino acid residues. Further screening of cDNA libraries allowed to identify more than 30 other Ca<sub>i</sub><sup>2+</sup> [70]. Moreover, high-affinity sensors for Na<sub>i</sub><sup>+</sup> are almost completely saturated at [Ca<sup>2+</sup>]<sub>i</sub> of 1  $\mu$ M. This allows identifying amino acid residues using <sup>45</sup>Ca binding assay. In contrast, molecular sensors

for monovalent ion may be presented by 3D protein structures formed with space-separated amino acid residues [27, 71]. Besides this, cellular functions are affected by monovalent cations when they act in the millimolar concentrations that make their detection with radioisotopes more complicate. As it was shown by Ono and coworkers,  $Na^+$  may interact with calpain  $Ca^{2+}$ -binding sites at the baseline level of  $[Ca^{2+}]_i$  (~100 nM). Thus, calpain functions as  $Ca^{2+}$ -dependent protease with  $K_{0.5}$  of 15 mM for  $Na^+$  [72]. Additional experiments should be performed to examine the role of  $Ca^{2+}$ -binding proteins as  $[Na^+]_i$  sensors involved in cellular responses evoked by hypoxia.

It is generally accepted that transcription is under the control of proteins interacting with specific response elements within 5'- and 3'-untranslated region (UTR). Considering this, we tried to find  $Na^+$  response element (NaRE) within c-Fos promoter. With the CRE and all other known c-Fos promoter transcription elements, we observed massive accumulation of endogenous c-Fos mRNA and immunoreactive protein in HeLa cells subjected to 6-h inhibition of  $Na^+, K^+$ -ATPase, but we did not find any significant increase in luciferase expression in ouabain-treated HeLa cells [33]. Negative results obtained in this study may be explained by the following hypotheses: (i) NaRE is located within the c-Fos 3'-UTR and/or introns. (ii) Elevation of  $[Na^+]_i/[K^+]_i$  ratio influences on gene expression through epigenetic modification of regulatory mechanism having a significant impact on various cellular functions, such the DNA, histones or nucleosome remodeling [73]. Importantly, the epigenetic mechanism of gene expression does not contribute to the regulation of L-luc transcription in the plasmid employed in our experiments [33]. (iii) More evidence indicates that gene activation or silencing is under the complex control of three-dimensional (3D) positioning of genetic materials and chromatin in the nuclear space (for review, see [74]). It may be proposed that gene transcription is affected by increased  $[Na^+]_i/[K^+]_i$  ratio through changing of the 3D organization of DNA-chromatin complex. These hypotheses will be verified in forthcoming studies.

Some studies have shown that epigenetic modulatory mechanism of histone methylation is a key process that helps cells to adapt to hypoxia [75]. Growing evidence shows that along with the 5'-UTR regulation by transcription factors, gene activation or silencing is controlled by 3D positioning of genetic materials and chromatin in nuclear spaces [74, 76]. The epigenetic regulation of 3D genome organization with considering the  $[Na^+]_i/[K^+]_i$  ratio and its role in gene silencing and activation is currently being examined in our laboratory.

Matrix metalloproteinases play an important role in pathophysiology of hypoxic chronic venous disease via their implication in the regulation of migration, proliferation and endothelium-dependent VSMC contraction [77]. We found that sustained elevation of the  $[Na^+]_i/[K^+]_i$  ratio resulted in ~fivefold elevation of Mmp28 metalloproteinase expression in rat VSMC [57]. The same procedure resulted in sevenfold elevation of the content of Nccp mRNA encoding natriuretic peptide precursor C [57]. NCCP is proteolytically processed to C-type natriuretic peptide (CNP), i.e., a selective agonist for the B-type natriuretic receptor whose role in cGMP-mediated vasorelaxation is well documented. We noted that in endothelial cells, modest long-term inhibition of the  $Na^+, K^+$ -ATPase causes ~sevenfold attenuation of expression of Edn encoding preproendothelin-1 that is proteolytically processed to the most pow-

erful endothelium-derived vasoconstrictor endothelin-1. We also observed ~10-fold elevation of the content of mRNA encoding ubiquitously derived vasodilator adrenomedullin (unpublished results). Do these  $[Na^+]_i/[K^+]_i$ -mediated transcriptomic changes contribute to the pathophysiology of hypoxic vascular disorders? Does partial dissipation of electrochemical gradients of monovalent cations seen in VSMC subjected to ischemia and glucose deprivation have an impact on the distinct regulation of systemic and pulmonary circulation under hypoxic conditions? We will address these questions to forthcoming studies.

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