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Review Article

The vascular Na,K-ATPase: clinical implications in stroke, migraine, and hypertension

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In the vascular wall, the Na,K-ATPase plays an important role in the control of arterial tone. Through cSrc signaling, it contributes to the modulation of Ca^{2+} sensitivity in vascular smooth muscle cells. This review focuses on the potential implication of Na,K-ATPase-dependent intracellular signaling pathways in severe vascular disorders; ischemic stroke, familial migraine, and arterial hypertension. We propose similarity in the detrimental Na,K-ATPase-dependent signaling seen in these pathological conditions. The review includes a retrospective proteomics analysis investigating temporal changes after ischemic stroke. The analysis revealed that the expression of Na,K-ATPase α isoforms is down-regulated in the days and weeks following reperfusion, while downstream Na,K-ATPase-dependent cSrc kinase is up-regulated. These results are important since previous studies have linked the Na,K-ATPase-dependent cSrc signaling to futile recanalization and vasospasm after stroke. The review also explores a link between the Na,K-ATPase and migraine with aura, as reduced expression or pharmacological inhibition of the Na,K-ATPase leads to cSrc kinase signaling up-regulation and cerebral hypoperfusion. The review discusses the role of an endogenous cardiotonic steroid-like compound, ouabain, which binds to the Na,K-ATPase and initiates the intracellular cSrc signaling, in the pathophysiology of arterial hypertension. Currently, our understanding of the precise control mechanisms governing the Na,K-ATPase/cSrc kinase regulation in the vascular wall is limited. Understanding the role of vascular Na,K-ATPase signaling is essential for developing targeted treatments for cerebrovascular disorders and hypertension, as the Na,K-ATPase is implicated in the pathogenesis of these conditions and may contribute to their comorbidity.

Introduction

Distinct expression and subcellular localization of Na,K-ATPase α isoforms in the vascular wall suggest their distinct functions

The Na,K-ATPase is a vital membrane transport protein that is present in all cells of the body [1]. It plays a critical role in the control of Na^+ and K^+ ion distribution over the cell membrane, as it is essential for, for example, maintaining the membrane potential and the secondary active transport that is important to build up the electrochemical gradient for other ions, including Ca^{2+}, Mg^{2+}, Cl^-, and H^+ [2]. The Na,K-ATPase consists of an α- and a β-subunit and associated regulatory proteins [3,4]. The catalytic α-subunit is responsible for ion transport and enzymatic activity of the Na,K-ATPase [5], while the chaperone β-subunit is essential for subcellular localization and have modulatory action for the enzyme including the regulation of affinity for Na^+ and K^+ [6]. Four different isoforms of the α-subunit exist with an unequal distribution throughout the body [1]. The α_1 isoform is expressed in all cells whereas the α_2, α_3, and α_4 isoforms demonstrate cell-type specific profiles [7]. The α_3 and α_4 isoforms are expressed in neurons and in the testes, respectively [8,9]. The α_2 isoform is mainly expressed in astrocytes and muscle tissues, including vascular smooth muscle cells [10,11].

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Interactions between the Na,K-ATPase and specific lipids and proteins in the plasma membrane result in the formation of lipid–protein complexes, influencing the organization of membrane microdomains and lateral mobility of membrane components, thereby modulating cell membrane fluidity [12–15]. Changes in membrane fluidity reciprocally impact the activity of the Na,K-ATPase itself, indicating a dynamic interplay between the enzyme and the cell membrane [14,16,17]. The Na,K-ATPase is also intricately regulated by the concentrations of its substrates, i.e., Na^+, K^+, and intracellular ATP [12]. Catecholamines that involve numerous intracellular signaling pathways, for example, protein kinases A, G, and C, tyrosine kinases, protein phosphatases and phospholipase have also been shown to modulate the activity of the Na,K-ATPase [12,18–20]. Furthermore, the actions of insulin, aldosterone, and carbachol involve changes in the subcellular distribution of the subunits of the Na,K-ATPase [13]. In this review, we will discuss how the Na,K-ATPase may potentially have implications for diseases such as stroke, migraine, and hypertension, and propose some similarities in their background mechanisms.

Cardiotonic steroids, including ouabain, digoxin, and digitoxin that bind to the α-subunit are well-known regulators of Na,K-ATPase activity [21]. Notably, the discovery of endogenous ouabain, and some other cardiotonic steroids that are synthesized by the body and use the Na,K-ATPase as a signaling receptor, has been made [22,23]. The different cardiotonic steroids share structural similarities containing a steroid core that can be glycosylated and a five- or six-membered lactone ring [24]. These functionalities affect the binding properties of specific cardiotonic steroids [21,25] although the high-affinity binding site for the cardiotonic steroids is the same [26]. Cardiotonic steroids inhibit the Na,K-ATPase by binding to the transmembrane domain of the α-subunit from the extracellular side and by stabilization of the outward-open phosphorylated E2 state, thus antagonizing K^+ binding [27]. Upon binding of cardiotonic steroids, the α isoform undergoes conformational changes that seems to be dependent on the type and number of substituents on the steroid core and the size of lactone ring of the cardiotonic steroid molecule [21]. Thus, the functional consequences of different cardiotonic steroids may vary, possibly because of different conformational changes they induce, although this remains to be validated [28]. In rodents, due to mutation in the high-affinity binding site for cardiotonic steroids, the α_1 isoform exhibits a lower sensitivity to cardiotonic steroids, including ouabain, requiring concentrations approximately thousand-fold higher than those necessary to effectively inhibit the α_2 and α_3 isoforms [27]. Hence, functional characteristics of the α_1 and α_2 isoforms can be pharmacologically differentiated with cardiotonic steroids [29–31].

Cardiotonic steroids, particularly ouabain, have a well-described pro-contractile effect on the heart and blood vessels [11,32,33]. In cardiomyocytes and vascular smooth muscle cells, the Na,K-ATPase α_2 isoform is restricted to specific areas of the plasma membrane in close proximity to the Na,Ca-exchanger (NCX) [29,34]. Both the Na,K-ATPase and the NCX are electrogenic membrane transporters. As the Na,K-ATPase pumps two K^+ ions into a cell in exchange for pumping out three Na^+ ions, it contributes to membrane hyperpolarization [35]. Accordingly, inhibition of the Na,K-ATPase depolarizes the membrane but the magnitude of the effect differs between the isoforms [4]. Thus, inhibition of the α_2 isoform (i.e., 1–10 μM ouabain) only slightly depolarizes the smooth muscle cell membrane by approximately 2–3 mV [36,], while further elevation of ouabain to 1 mM inhibits all isoforms of the Na,K-ATPase [4] leading to further depolarization by approximately 5 mV (Figure 1A) [37]. The minor changes in membrane potential observed in the presence of ouabain below 1 mM may not be anticipated to exert a significant impact on the voltage-gated Ca^{2+} influx and thus arterial contraction [36,37–40].

The NCX, in its conventional configuration, exchanges 3 Na^+ ions with 1 Ca^{2+} ion and has a reversal potential positive to the resting membrane potential of smooth muscle cells in the arterial wall (Figure 1B) [43]. Thus, under resting conditions, the NCX is expected to transport Ca^{2+} out of the cell [43]. However, a slight elevation of $[Na^+]_{in}$ shifts the reversal potential leftwards, even below the resting membrane potential in smooth muscle cells, which is typically relatively depolarized and reported at approximately -45 – -55 mV [41,42]. This will first reduce the Ca^{2+} extrusion by the NCX and then, when the reversal potential is more negative than the membrane potential, change the NCX activity to a reversal mode that transports Ca^{2+} into the cell (Figure 1B) [43,44].

The colocalization of the Na,K-ATPase α_2 isoform with the NCX may facilitate efficient transport of Ca^{2+} out of the cell through the NCX that utilizes the locally restricted Na^+ gradient generated by the Na,K-ATPase [45] (Figure 2). Based on this isoform-specific colocalization, it has been suggested that the pro-contractile action of cardiotonic steroids is related to elevation of intracellular Ca^{2+} load [43,46]. The proposed mechanism stems from the inhibition of the Na,K-ATPase, which consequently prevents the active extrusion of Na^+ ions and leads to secondary elevation of intracellular Ca^{2+} load to the sarcoplasmic reticulum by the Sarcoplasmic/Endoplasmic Reticulum Ca^{2+}- ATPase [47]. The intracellular Ca^{2+}, derived from the extracellular space, is normally extruded in exchange of Na^+ by the NCX and through the sarcolemmal Ca^{2+}-ATPase. However, because of combined effect of membrane depolarization and intracellular Na^+ elevation, the potential for extrusion of Ca^{2+} by the NCX is reduced upon inhibition of the

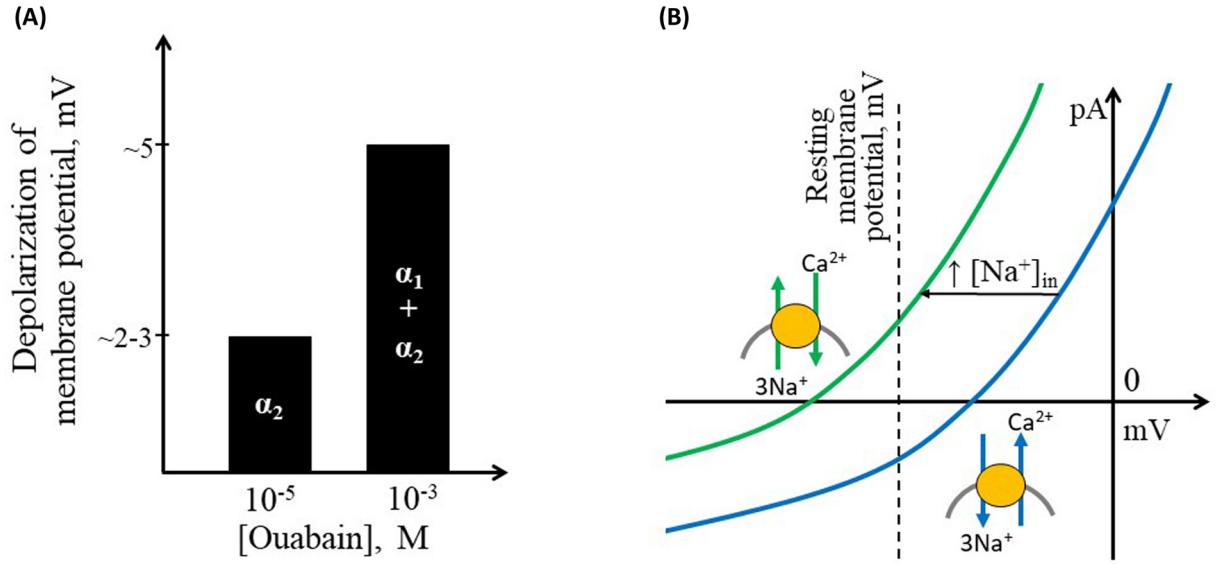

Figure 1. Schematic presentation of key electrogenic properties of different vascular isoforms of the Na,K-ATPase and the Na,Ca-exchanger (NCX)

(**A**) In the rodent vasculature, ouabain in concentration of 10^{-5} M almost completely inhibits the Na,K-ATPase α_2 isoform [4] leading to approximately 2–3 mV depolarization, while elevation of ouabain to 10^{-3} M further depolarizes cells with 5 mV [36,37]. (**B**) Current–voltage relationship for the NCX. The reversal potential of NCX in configuration 3 Na^+:1 Ca^{2+} at a normal ion distribution over the membrane ($[Na^+]_{in}$ 10 mM, intracellular $[Ca^{2+}]_{in}$ 200 nM, $[Na^+]_{out}$ 144 mM, $[Ca^{2+}]_{out}$ 1.6 mM) is estimated to be −26 mV (blue curve). This is positive to the resting membrane potential of vascular smooth muscle cells, which is usually reported at approximately -55 mV [41,42] in peripheral arteries and −45 mV in cerebral arteries [11]. This enables extrusion of Ca^{2+} from the cell, i.e., forward mode of the NCX activity [43]. However, even a slight elevation of $[Na^+]_{in}$ may shift the reversal potential of NCX to a value negative to the cell resting potential (e.g., change of $[Na^+]_{in}$ from 10 to 15 mM moves the NCX reversal potential to −59 mV; i.e., a leftward shift of the curve indicated with green color), leading to the reverse mode of NCX transport, i.e., Ca^{2+} will be transported to the intracellular space [43].

Na,K-ATPase by cardiotonic steroids. This increases the Ca^{2+} concentration in the cytosol that is then stored in the sarcoplasmic reticulum and contributes with greater Ca^{2+} release upon agonist stimulation [45,48].

Digoxin is a Na,K-ATPase inhibitor that is used in clinical settings to regulate cardiac frequency and potentiate the cardiac contractility [43]. However, unlike ouabain, digoxin does not have the same potentiating effect on vascular contraction, despite its similar inhibition of the ion transport [11,54]. The specific reason for these different functional effects remains to be determined but it may imply that the modulation of arterial contractility by the Na,K-ATPase cannot solely be attributed to the direct inhibition of ion pumping activity [10,55]. In discussing the importance of Na,K-ATPase for diseases, it is therefore crucial to consider its role as an intracellular signaling hub.

Na,K-ATPase-dependent cSrc signaling regulates Ca^{2+} sensitivity in smooth muscle cells

To elucidate the role of Na,K-ATPase for the pathologies, it is important to understand how the Na,K-ATPase signals in the cells. In this section, we will therefore summarize how signaling from the Na,K-ATPase may occur. Apart from controlling intracellular Ca^{2+}-signaling, the Na,K-ATPase has been suggested to regulate several other intracellular signaling pathways [45,56–59] in a cation-independent manner [49, 60–65]. Two Na,K-ATPase-dependent signaling pathways has been implicated in cell proliferation, these are the EGFR/cSrc/Ras/Erk1/2 and the PI3KA1/Akt pathways [66–68] [EGFR, epidermal growth factor receptor; Erk1/2, p44/42 mitogen-activated protein kinase; PI3KA1, phosphatidylinositide 3-kinase 1A]. Thus, activation of the PI3KA1/Akt signaling has been implicated in ouabain-induced cardiac hypertrophy [68,69]. Whether this pathway is dependent on cSrc kinase signaling remains controversial [56,66,68] and its role in the vascular wall is not yet elucidated. The Na,K-ATPase-regulated PLCγ/InsP_3R signaling pathway has been suggested to modulate Ca^{2+} release from sarcoplasmic reticulum [45,70] [PLCγ, phospholipase Cγ; InsP_3R, inositol trisphosphate receptor]. Hence, there are several signaling pathways under regulation of

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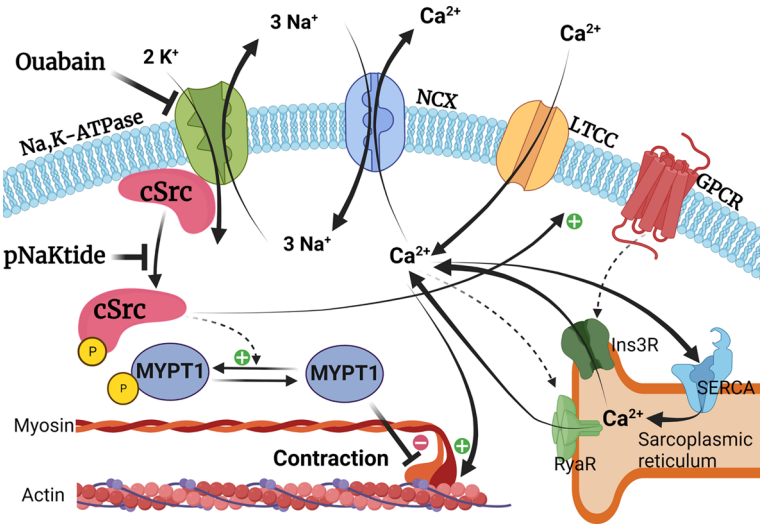

Figure 2. Signaling pathways behind potentiation of vascular smooth muscle contraction by ouabain

The Na,K-ATPase generates the electrochemical gradient for Na^+ ions, driving Ca^{2+} extrusion through the Na,Ca-exchanger (NCX) [45]. Upon binding of ouabain, the Na,K-ATPase pumping is impaired, reducing the NCX driving force and increasing intracellular Ca^{2+} concentration [43,46]. This surplus Ca^{2+} is then loaded into the sarcoplasmic reticulum by the sarcoendoplasmic reticulum Ca^{2+} ATPase (SERCA) [45,48]. Subsequently, more Ca^{2+} is released from the sarcoplasmic reticulum via the inositol trisphosphate receptors (Ins3R) upon G-protein coupled receptor (GPCR) stimulation and/or as a Ca^{2+}-induced Ca^{2+} release through ryanodine receptors (RyaR) resulting in potentiated smooth muscle contraction [45,48]. Ouabain also induces cSrc kinase phosphorylation, ultimately increasing the sensitivity of the contractile machinery to intracellular Ca^{2+} [11,39]. This occurs through the phosphorylation of myosin phosphatase target subunit 1 (MYPT1), leading to the inhibition of the myosin light chain phosphatase, resulting in Ca^{2+} sensitization [10,11,39,49,50]. A synthetic peptide inhibitor, pNaKtide [51], inhibits cSrc kinase activation and thus, reduces smooth muscle Ca^{2+} sensitivity [11]. The cSrc kinase signaling may also control intracellular Ca^{2+} concentration through the phosphorylation of L-type calcium channels (LTCC), leading to increased Ca^{2+} influx [52,53].

the Na,K-ATPase that may affect vascular tonus but their function in vascular smooth muscle cells needs to be validated. Most investigations have focused on the Na,K-ATPase-dependent pathways downstream from cSrc kinase [49, 61–65]. The Na,K-ATPase α_1 isoform has been demonstrated to contain a cytosolic inhibitory cSrc-binding domain [62,71,72]. Binding of ouabain to the Na,K-ATPase [61] leads to release of the Na,K-ATPase-bound cSrc kinase and its auto-phosphorylation at Tyr-418 that activates the enzyme [36,60,63,73,74]. Importantly, the fact that cSrc is activated upon ouabain binding to the Na,K-ATPase is well-established [75,76], but the mechanism behind it remains controversial as some studies failed to demonstrate the physical interaction between the Na,K-ATPase and cSrc kinase [77,78]. Hence, it has been proposed that cSrc autophosphorylation is a result of localized increase in intracellular ATP concentration because of inhibited activity of the Na,K-ATPase [79]. Importantly, binding of digoxin to the Na,K-ATPase failed to activate cSrc kinase in rat smooth muscle cells *ex vivo* [80] arguing against indirect cSrc activation via the elevated ATP concentration. The reason for these different actions of the two cardiotonic steroids is unclear, although the effect of different conformational changes in the Na,K-ATPase transmembrane complex upon binding these two cardiotonic steroids has been speculated [21,39]. These different conformation changes can be a result of the variable structural and thus, binding characteristics of the different cardiotonic steroids, as discussed above.

The phosphorylation level of cSrc kinase is inversely correlated with the expression of the α_1 isoform [11,72,81,51]. Whether other α isoforms than the α_1 isoform are also capable of modulating cSrc kinase signaling is currently a matter of controversy [39,82]. In the studies on epithelial cells, a distinct role of the α_1 isoform is suggested [83]; however, these cells lack the native expression of other Na,K-ATPase isoforms [4]. In contrast, the Na,K-ATPase-dependent cSrc signaling in the rodent vascular wall was activated by ouabain concentrations that specifically act on the α_2 isoform suggesting its role in this signaling [11,36,39,49,80]. Furthermore, both the α_1 and α_2 isoforms were shown to interact with cSrc kinase human skeletal muscle cell culture [84]. It might, however, also be an indirect effect of the α_2 isoform on cSrc activation as the Na,K-ATPase α_1 isoform inhibits cSrc in its E1 conformation [62], which depends

on Na^+ in the cytosol [85]. Ouabain inhibition of the Na,K-ATPase α_2 isoform may therefore affect the localized Na^+ concentration and thus, initiate cSrc activation from the α_1 isoform. Future studies need to address these possibilities.

The ouabain-induced potentiation of the constriction of cerebral arteries [11] and mesenteric small arteries [39] is related to the amplification of cSrc signaling. Moreover, cSrc kinase contributes to endothelin-1-induced vasoconstriction of isolated rabbit basilar arteries [49,86], and is necessary for the myogenic response of rat cerebral arteries [87]. The cSrc kinase activation is shown to lead to myosin phosphatase target subunit 1 (MYPT1) phosphorylation in vascular smooth muscle cells of cerebral arteries [11], as well as in arteries of various other tissues [10,39,49,88,50]. However, the precise signaling mechanism between cSrc and MYPT1 has not yet been fully elucidated [55] as cSrc tyrosine kinase cannot directly phosphorylate MYPT1 threonine residuals that were shown involved in this signaling [89]. MYPT1 is an enzyme, which regulates the phosphorylation of myosin light chain phosphatase and thereby the sensitivity of the contractile machinery of vascular smooth muscle cells to intracellular Ca^{2+} (Figure 2) [55,90]. This phenomenon is known as '*Ca^{2+} sensitization*' and implies a potentiation of smooth muscle contractile state due to increased sensitivity of myofilaments to intracellular Ca^{2+} without any change in Ca^{2+} concentration [91]. It thus seems likely that ouabain consequent to binding to the Na,K-ATPase modifies cSrc signaling in smooth muscle and enhances the sensitivity of the contractile apparatus to Ca^{2+}.

This has indeed been supported by experiments directly showning that ouabain potentiates the smooth muscle cell contraction via Ca^{2+} sensitization [11,39]. In accordance with the concept of sensitization of vascular smooth muscle cells to intracellular Ca^{2+} [91,92], ouabain-potentiated vasoconstriction is associated with minor or no change in intracellular Ca^{2+} [11,39,49,93]. Moreover, the source of intracellular Ca^{2+} and the type of contractile stimuli are not essential for ouabain-induced potentiation of contraction via the Ca^{2+} sensitization [11]. Thus, ouabain potentiates the contraction initiated by different agonists (e.g., noradrenaline, thromboxane, and endothelin) and K^+-induced depolarization. The K^+-induced depolarization and contraction depends on Ca^{2+} influx from the extracellular space via the voltage-gated Ca^{2+} channels, while the contraction upon stimulation of G-protein-coupled receptors depends on both Ca^{2+} influx and Ca^{2+} release [94]. This supports the suggested primary role of the pro-contractile ouabain-induced cSrc activation, that is Ca^{2+} sensitization via MYPT1 phosphorylation, although an effect of ouabain on other pathways in the vascular wall as discussed above cannot be excluded. Accordingly, it has been reported that phosphorylation by cSrc potentiates the activity of voltage-gated L-type Ca^{2+} channels [52,53]. Moreover, in non-muscular cells, cSrc was shown to potentiate the Ca^{2+} release via an activation of the inositol trisphosphate receptors in the sarcoplasmic reticulum membrane and to suppress the activity of voltage-gated K^+ channels leading to further depolarization and voltage-dependent Ca^{2+} influx [,95] (Figure 2). Hence, there are multiple signaling pathways in which binding of ouabain to the Na,K-ATPase may potentiate the contraction of vascular smooth muscle cells but the involvement of Ca^{2+} sensitization seems to have prominent action (Figure 2).

Involvement of the Na,K-ATPase in ischemic stroke pathogenesis

Role of vascular dysfunction in futile recanalization

The treatment of ischemic stroke has been revolutionized by the development and implementation of endovascular thrombectomy techniques [96]. However, 54% of patients with successful macrovascular recanalization after thrombectomy are either disabled to a degree requiring external assistance or deceased 90 days after the stroke, a phenomenon known as futile recanalization [97,98]. Several mechanisms have been suggested to account for futile recanalization including vascular dysfunction [99,100]. Hence, futile recanalization has been attributed to impaired microvascular reperfusion, observed in approximately 25% of ischemic stroke patients receiving endovascular thrombectomy [101]. Preclinical studies suggest that microvascular failure after reperfusion is associated with undesirable contraction of capillary pericytes in the peri-ischemic penumbra [102] and in the ischemic core [103–106]. Although spreading depolarizations during arterial occlusion and hypoxic injury have been suggested to account for capillary pericyte contraction, the molecular mechanism behind the contraction remains largely unknown [103–108]. Importantly, constriction or vasospasm of upstream pial arteries [109] and parenchymal arterioles [100,110,111] have also been suggested to be involved in futile recanalization. Changes in arterial function following ischemic stroke have received relatively little attention compared with hemorrhagic stroke [112]. Acquiring a more comprehensive understanding of post-stroke vascular dysfunction could be of great importance in preventing infarct growth associated with secondary brain injury in ischemic stroke patients [112,113], especially given that all previous clinical trials aimed at preventing such injury have been unsuccessful [114].

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Association between the Na,K-ATPase-dependent cSrc signaling and post-stroke vasospasm

Previous studies in rats indicate an increased vascular tone of rat arterioles after ischemic stroke [100,115]. This increased arteriolar tone is not associated with changed smooth muscle cell membrane potential nor changed intracellular Ca^{2+} concentration compared with rats undergoing sham surgery [100]. Accordingly, the excessive tonus of arteries and arterioles following stroke is suggested to be caused by elevated sensitivity of the contractile machinery to Ca^{2+} (Figure 2) [100,109]. Measurements of intracellular Ca^{2+} changes in post-stroke rat arterioles demonstrated stronger contraction at the same Ca^{2+} concentration in comparison with matched sham operated group suggesting the Ca^{2+} sensitization [100]. Further research is required to clarify if post-stroke capillary failure is also associated with increased Ca^{2+} sensitivity of contractile pericytes [102,103]. In the following, it will be discussed whether the Na,K-ATPase-dependent phosphorylation of cSrc kinase is important for the increased sensitivity of arteries and arterioles to intracellular Ca^{2+} after stroke.

The vasoconstriction of pial arteries downstream from the occlusion after reperfusion is associated with increased Na,K-ATPase-dependent phosphorylation of cSrc kinase [109]. Increased vascular tone following stroke could arise from enhanced cSrc/PLCγ/InsP_3R signaling, triggering an increased release of Ca^{2+} from the sarcoplasmic reticulum [45,70]. However, given that the intracellular Ca^{2+} concentration in vascular smooth muscle cells remains similar between stroke and control rats [100], this mechanism appears unlikely. Conversely, increased Ca^{2+} sensitization of the contractile machinery after stroke may be mediated through activation of cSrc by phosphorylation resulting in potentiation of smooth muscle contraction [11,39,49] even at unchanged intracellular Ca^{2+} concentration as discussed above (Figure 2) [100]. This suggests the cSrc-associated Ca^{2+} sensitization as a novel treatment target to prevent post-stroke vasospasm. In fact, cSrc kinase inhibition has been studied in rodent models of experimental stroke. Pharmacological inhibition of Src family tyrosine kinases with relatively specific inhibitors, PP1 or PP2, is associated with reduced cerebral infarctions as well as improved neurological outcomes [116–119]. The inhibition of cSrc kinase decreases smooth muscle cell Ca^{2+} sensitivity, leading to reduced vasoconstriction of isolated mouse middle cerebral arteries [11]. Accordingly, the positive outcome resulting from PP1-induced Src family kinase inhibition following stroke is accompanied by an increase in cerebral perfusion when compared with vehicle-treated controls that also underwent stroke [116]. Since this study employed magnetic resonance imaging to quantify cerebral blood flow, the spatial resolution was insufficient to investigate the distinct contributions of different cerebrovascular beds, and other mechanisms of futile reperfusion, for example, a disrupted blood–brain barrier cannot be excluded [116–119]. Other potential reasons for the favorable stroke outcome following cSrc inhibition warrant careful consideration. Notably, the Na,K-ATPase-dependent cSrc/Ras/Erk1/2 signaling pathway has been proposed to elicit excessive generation of reactive oxygen species within the mitochondria [60,73,74], a pivotal mechanism in the pathophysiology of ischemic stroke [120]. Therefore, the improved stroke outcome linked to cSrc inhibition could potentially stem from the prevention of excessive reactive oxygen stress, thereby reducing tissue damage following stroke.

Down-regulation of the Na,K-ATPase after stroke reperfusion

To test whether increased phosphorylation of cSrc kinase after stroke reperfusion is associated with the changes in abundancy of the Na,K-ATPase, we assessed a previously published proteomics dataset [121] (Reprinted with permission, Wen et al., 2019, *J. Proteome Res.*, copyright © 2019 American Chemical Society). We compared the proteome of rat brains subjected to a 2-h filament-induced middle cerebral artery stroke with the proteome of matched control rats that underwent sham surgery. Samples of the rat brain were collected from the stroked hemisphere at three time points (1, 7, and 14 days) after stroke reperfusion or sham surgery to investigate the temporal changes in protein expression. Groups were compared using two-way ANOVA with Turkey's post-hoc test. The expression of the Na,K-ATPase α_1, α_2, and α_3 isoforms were reduced at all three time points after reperfusion compared with the sham group (Figure 3A–C). The expression of the α_1 and α_3 isoforms were lower at the 14 days follow-up compared with the 1- and 7-day follow-ups. Intriguingly, the expression of cSrc in the same proteomics dataset was up-regulated at the 7-day follow-up after stroke compared with the sham group (Figure 3D). Two other Src family tyrosine kinases, Lyn and Hck, were also detected by proteomics, but their expression was not affected by stroke ($P>0.5$). Importantly, this analysis is made on a bulk proteomics dataset [121]. A spatially restricted assessment is necessary to investigate whether cSrc up-regulation associated with the Na,K-ATPase down-regulation is localized to brain mural cells, i.e., contractile pericytes and smooth muscle cells [122].

The implication of altered cSrc kinase signaling after stroke, while the expression of other Src family kinases remains unchanged, is consistent with the findings of a prior study in a mouse model of ischemic stroke [116]. In that study, it was shown that homozygous global knockout of the cSrc gene is associated with reduced infarct size, whereas

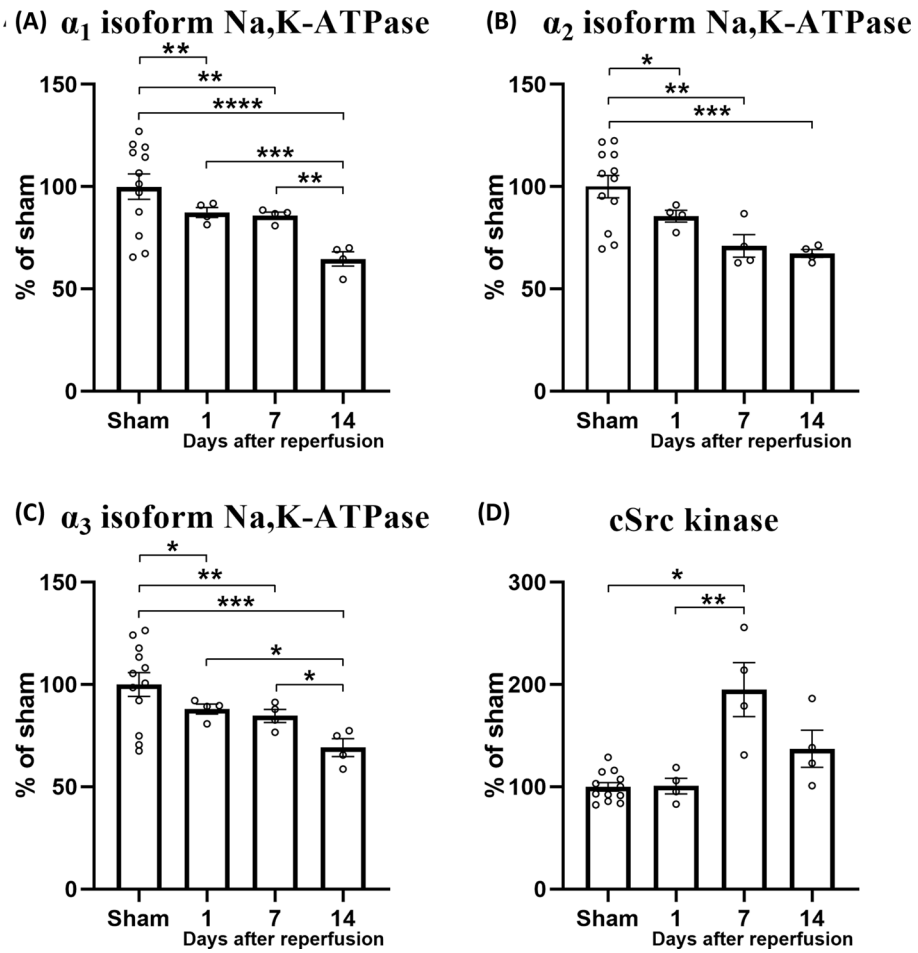

Figure 3. Stroke led to down-regulated expression of the Na,K-ATPase α isoforms and up-regulation of cSrc kinase expression

Proteomics analysis of brain tissue of filament-induced middle cerebral artery stroke and sham-operated rats (n=12). Brain tissue was sampled 1, 7, or 14 days after stroke ($n = 4$ for each follow-up). The expression of Na,K-ATPase α_1 isoform was down-regulated after stroke compared with sham-operated rats (**A**). The expression of the α_1 isoform was lower at the 14-day follow-up compared with the 1- and 7-day follow-up. The expression of the α_2 (**B**) and α_3 isoforms (**C**) were also reduced after stroke compared with sham-operated rats. The expression of the α_3 isoform was more reduced at the 14-day follow-up compared with the 1- and 7-day follow-up (**C**). The expression of cSrc kinase was increased at the 7 days follow-up compared with the sham-operated group and the 1 day follow-up (**D**). Reprinted with permission from previously published dataset by Wen et al., *J. Proteome Res*. (2019) [121], Copyright © 2019 American Chemical Society. Data were normalized to the sham-operated group that was set to 100%. Groups were compared using two-way ANOVA with Turkey's post-hoc test. *, **, ***, ****, $P<0.05$, 0.01, 0.001, 0.0001.

homozygous knockout of another Src family kinase member, Lyn, results in a similar infarct size to that of wild-type mice [116]. These results suggest that cSrc plays a specific role in the pathology of stroke. The proteomics dataset [121] that we assessed cannot provide data on the protein phosphorylation level. Further, the dataset is not cell-type specific and the contribution of specific cell types in the brain to changed expressional levels cannot be differentiated. Nevertheless, these proteomics data strongly suggest that all three Na,K-ATPase α isoforms expressed in the brain are down-regulated after stroke. At the same time, it appears that cSrc kinase, which is normally dephosphorylated in the presence of active Na,K-ATPase in the cerebral vasculature, may become phosphorylated, which is consistent with previous observations in a mouse model of ischemic stroke.

Reduced activity of the Na,K-ATPase has been reported in various experimental models of hypoxic and ischemic pathologies [123,124]. Accordingly, hypoxia leads to reduced Na,K-ATPase expression and activity in isolated vascular smooth muscle cells of rat basilar arteries [125]. The Na,K-ATPase is one of the most ATP-consuming proteins

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in the body [126–128]. Hence, reduced activity or expression of the Na,K-ATPase under conditions with restricted supply of nutrients or oxygen may be an effective strategy to balance intracellular energy [129]. This fine-tuned balance may be regulated by the release of endogenous ouabain [130,131]. Hypoxia leads to increased level of oxidative stress, which has also been suggested to regulate the Na,K-ATPase through carbonylation of the α_1 isoform [132,133]. Importantly, oxidative stress-mediated carbonylation of the α_1 isoform increases the phosphorylation of cSrc [132,133]. Amplified cSrc signaling is associated with detrimental impact on mitochondrial respiration [60,74,93], which may further increase mitochondrial generation of reactive oxygen species and thus, a vicious circle is established [93,129,134]. In the vascular smooth muscle cells, the vicious circle resulting in impaired Na,K-ATPase and amplified cSrc kinase activity may lead to exaggerated vasoconstriction through the mechanisms discussed above. In strong support of this, pNaKtide, a specific inhibitor of the Na,K-ATPase-dependent cSrc activation [11,36,51,93,135], may break the vicious cycle. pNaKtide is a 20-amino acid peptide that mimics cSrc-binding sequence in the N-terminus of Na,K-ATPase and inhibits cSrc in an ATP-independent manner [51]. It remains, however, to be tested whether pNaKtide treatment can prevent post-stroke vasospasm and improve the outcome after stroke.

Mice with heterozygous knockout of the Na,K-ATPase α_1 isoform have larger cerebral infarct size after middle cerebral artery occlusion than wild-type mice [136]. Further, reduced expression of both α_1 and α_2 isoforms in cerebral arteries in mice is associated with amplified cSrc kinase signaling and increased tone of the middle cerebral artery [11]. As discussed above, the role of α_2 isoform in cSrc signaling is controversial [39,83]; thus, the isoform-specific contribution of the Na,K-ATPase remains to be elucidated. A further reduction in the Na,K-ATPase α isoform expression and cSrc activation following stroke, as the proteomics analysis suggested (Figure 3A–C), may lead to vasospasm. This implies that cSrc-dependent excessive vasoconstriction may contribute to the poor stroke outcome in mice with heterozygous knockout of the α_1 isoform [136]. Whether the reduction in the Na,K-ATPase α_2 isoform will also have similar consequences for stroke outcome remains to be studied.

The implication of the Na,K-ATPase in pathophysiology of migraine with aura

Reduced expression or pharmacological inhibition of the Na,K-ATPase increases susceptibility for spreading depressions

Migraine with aura is associated with a drop in blood flow in the affected hemisphere. This is also the case for the peri-ischemic tissue after ischemic stroke. It is generally accepted that the drop in blood flow is associated with spreading depression, i.e., cortical and peri-ischemic spreading depression in migraine aura and ischemic stroke, respectively [137]. The spreading depression is represented as a wave of depolarization propagating through the neuronal tissue across the cortex with a speed of ∼3 mm/min [102,138,139]. During spreading depression, both neuronal and glial cells undergo depolarization, resulting in a shift of the transmembrane ion balance [137]. This shift involves a substantial increase in extracellular K^+ concentration [140] due to the efflux of K^+ as the repolarizing current [137,141]. It is, therefore, of relevance to understand the role of the Na,K-ATPase in migraine with aura and the associated spreading depression. As the concentration of extracellular K^+ rises, the cell membrane depolarizes [142]. Consequently, voltage-gated cation channels on neuronal membranes become more susceptible to opening, allowing influx of Ca^{2+} and Na^+ ions, which further depolarizes the membrane potential [137,142].

The role of the Na,K-ATPase in spreading depression is suggested from the finding that ouabain induces spreading depression in rat brain slices [143,144] and *in vivo* in mice [145]. The proposed mechanism for ouabain's ability to induce spreading depression is through the inhibition of Na,K-ATPase ion-pumping activity [146]. Hence, ouabain may lead to an elevation of extracellular K^+ concentrations resulting in membrane potential depolarization and thus initiation of the spreading depression [146,147]. Importantly, spreading depressions may be triggered by low ouabain concentrations that selectively inhibit the Na,K-ATPase α_2 and α_3 isoforms in rodents without any significant effect on the α_1 isoform [31,148]. On strong support of this, heterozygous knockout [149] or small hairpin RNA (shRNA)-induced knockdown of the α_2 isoform [145] is associated with increased susceptibility to experimental spreading depression in mice. Conversely, heterozygous knockout of the α_3 isoform is associated with lower susceptibility to spreading depression whereas heterozygous knockout of the α_1 isoform does not change spreading depression susceptibility [149]. The reason for this difference is unclear but possibly the different expression pattern of the isoforms leads to different extracellular K^+ clearance in the brain parenchyma. The α_2 isoform is expressed in astrocytes [4,7], which may be specifically important for K^+ clearance [150,151]. Further, in contrast with the Na,K-ATPase α_1 isoform, both α_2 and α_3 isoforms are not saturated at normal K^+ concentrations, and therefore have

a capacity to further increase their activity upon elevation of extracellular K^+ [152]. These observations are of high clinical relevance since mutation in the α_2 isoform is linked to familial hemiplegic migraine.

Familial hemiplegic migraine-associated mutation of the Na,K-ATPase is associated with exaggerated constriction of cerebral arteries

Familial hemiplegic migraine type 2 (FHM2; OMIM No. 602481) is a rare subtype of migraine with aura associated with mutation in the *ATP1A2* gene encoding the Na,K-ATPase α_2 isoform [153–155]. Some *ATP1A2* mutations, including the G301R missense mutation [11,74,156–158], lead to reduced expression of the α_2 isoform whereas other mutations do not change the expressional level [159] but probably affect the function or regulation of the Na,K-ATPase. In line with the α_2 isoform knockout studies [145,149], mice carrying FHM2-associated mutations of the α_2 isoform exhibit increased susceptibility for cortical spreading depolarization [160–162]. The α_2 isoform is important for generating the driving force for extracellular space clearance by astrocytes, including reuptake of glutamate and K^+ released upon neuronal excitation [150,151]. Consistent with this mechanistic view, astrocytic glutamate reuptake was shown to be reduced in two different murine models of FHM2 [157,160].

It is often overlooked in research on FHM2 [153,163] that the α_2 isoform is expressed not only in glial cells but also in the endothelium and smooth muscle cells of the vascular wall [11,29,164]. As discussed above, the α_2 isoform plays a key role for vascular function, and it is therefore possible that cerebrovascular abnormalities may contribute to the migraine pathophysiology of FHM2 [10,46,55,165]. Indeed, mice carrying the FHM2-associated G301R mutation, ${\alpha_2}^{+/G301R}$ mice, show abnormal neurovascular coupling [166], and excessive vasoconstriction of cerebral arteries associated with cerebral hypoperfusion [11]. Curiously, the increased vasoconstriction is associated with a smaller increase in agonist-induced intracellular Ca^{2+} concentration in cerebrovascular smooth muscle cells of ${\alpha_2}^{+/G301R}$ compared with wild-type mice [11]. This implies that increased cerebrovascular contractility in ${\alpha_2}^{+/G301R}$ mice cannot be explained by elevation of intracellular Ca^{2+} concentration in vascular smooth muscle cells of ${\alpha_2}^{+/G301R}$ mice [11]. Similar to that observed in arterioles after stroke [100], this implies that the increased vasoconstriction of cerebral arteries in ${\alpha_2}^{+/G301R}$ mice is attributed to augmented Ca^{2+} sensitization in smooth muscle cells that is associated with amplified Na,K-ATPase-dependent phosphorylation of cSrc kinase and MYPT1 (Figure 2) [11]. Indeed, it was shown that cSrc phosphorylation was increased in cerebral arteries of ${\alpha_2}^{+/G301R}$ mice and acute cSrc inhibition abolished the difference in vascular responses between ${\alpha_2}^{+/G301R}$ and wild-type mice [11]. Therefore, it seems likely that the cSrc pathway is important for the pathophysiology of FHM2. It is possible that other Na,K-ATPase-dependent signaling pathways affecting cell proliferation, i.e., EGFR/Src/Ras/ERK and PI3KA1/Akt pathways, may contribute to the altered cerebrovascular function in FHM2. In fact, middle cerebral arteries of ${\alpha_2}^{+/G301R}$ mice exhibit increased vascular wall thickness and media-to-lumen ratio compared with wild-type mice [11].

Migraineurs with aura exhibit interictal cerebral hypoperfusion

Whether the enhanced Ca^{2+} sensitivity of cerebrovascular smooth muscle cells is associated with increased vasoconstriction in other types of migraine with aura than FHM2 has yet to be studied. However, previous studies have reported cerebral hypoperfusion of migraineurs with aura in the interictal period [167–169]. Several contractile factors may provoke cerebral hypoperfusion in migraineurs with aura. For example, one study found that the plasma level of thromboxane A_2, a potent vasoconstrictor, is higher in migraineurs during the interictal period than in patients with tension-type headache or healthy individuals [170]. Other studies have proposed that migraineurs with aura have elevated plasma level of another vasoconstrictor, endothelin-1, in the interictal period [171,172]. Interestingly, it has also been reported that migraineurs have an increased concentrations of endogenous ouabain in the blood in the interictal period [173], suggesting that the inhibition of the Na,K-ATPase may lead to exaggerated vasoreactivity [39,174]. Importantly, the elevation of endogenous ouabain is seen in the blood but not in the cerebrospinal fluid [173]. Due to its hydrophilic structure, ouabain cannot easily cross the blood–brain barrier [175], therefore deviding the body's endogenous ouabain in two compartments, i.e, periphery and the brain [45]. This suggests that the action of increased endogenous ouabain in the blood of migraineurs [173] may be addressed to the cerebral vasculature from the circulation site, and not to neurons and astrocytes located behind the blood–brain barrier. However, the specific extent to which ouabain from the blood can pass the blood–brain barrier remains to be studied. The finding of elevated levels of endogenous ouabain in migraineurs [173] calls for further research to clarify the contribution of Na,K-ATPase-dependent disturbances in cerebrovascular function in migraine with aura.

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The role of the Na,K-ATPase in hypertension

Endogenous cardiotonic steroid-like compounds

The first indications that the body may produce a cardiotonic steroid stems from investigations of volume-dependent hypertension [176]. Nearly half a century ago, a reduced pumping activity of the Na,K-ATPase in the vascular wall of hypertensive dogs was reported [177]. This led to the proposal of the presence of an endogenous compound similar to cardiotonic steroids [178]. Moreover, a strong correlation between the capacity of blood plasma to inhibit the Na,K-ATPase and blood pressure measured in patients with hypertension was discovered [179]. As a result, an endogenous cardiotonic steroid was isolated from human serum and was claimed to be structurally indistinguishable from ouabain [180]. As its structure is still debated, it is often termed as the endogenous ouabain-like compound [181], but it is proposed to be chemically identical to the original plant-derived alkaloid ouabain [182]. Later, other endogenous cardiotonic steroids, for example, marinobufagenin and telocinobufagin, were also found in the human body [183]. The binding site for cardiotonic steroids in the Na,K-ATPase is highly conserved through evolution [21], with only few exceptions [184]. This might suggest that ouabain is physiologically (and pathophysiologically) important and consistent with a possible role in hypertension [185].

Ouabain is synthesized in the zona glomerulosa cells of the adrenal cortex as other adrenal steroids [186,187]. Hypothalamus is also suggested as a site for production of endogenous ouabain [188,189], which may play a central neuromodulatory role leading to excitation of the central sympathoexcitatory pathways [190,191]. Two separate secretion sites and ouabain's impermeability over the blood-brain barrier [175], suggest the existence of two signaling systems for endogenous ouabain [45].

Hydroxycholesterol, pregnenalone, and progesterone have been shown to increase the secretion of endogenous ouabain, possibly acting as precursors in its biosynthetic pathway [192,193]. Interestingly, the use of the oral contraceptive, progesterone, was previously shown in migraineurs with aura to be associated with increased prevalence of ischemic stroke [194–197]. However, a direct link between the level of endogenous ouabain and ischemic stroke has not yet been investigated. The synthesis of ouabain follows a similar pathway as aldosterone [198]. The exact mechanisms and precursors directly involved in the biosynthesis of ouabain remain, however, unclear. Several factors have been suggested to increase the production of endogenous cardiotonic steroids, including blood pressure elevating hormones, drugs, and interventions such as angiotensin II, vasopressin, adrenocorticotropic hormone (ACTH), hypoxia, physical activity, and stress [130,199–201]. There are several lines of evidence to suggest that ouabain is synthesized and secreted by the adrenal cortex. First, ouabain is found at high concentrations in the adrenal cortex [180,189]. Second, bovine adrenocortical cells secrete ouabain in amounts greater than their storage capacity under *in vitro* conditions [202]. Third, the concentration of ouabain in adrenal veins is significantly higher than in arterial plasma [203]. Moreover, some adrenal cortex tumors are associated with excessive production and secretion of ouabain [204]. Consistently, administration of anti-ouabain antibodies leads to adrenal cortex enlargement in rats, further implicating the adrenal gland as a source of ouabain [205].

The majority of endogenous ouabain detection studies are based on blood using immunoassays, for example, enzyme-linked immunosorbent assay (ELISA) and radioimmunoassay [206]. The results of these methods are supported by high-resolution mass spectrometry and nuclear magnetic resonance spectroscopy [180]. However, the validity of endogenous ouabain detection is still questioned [181]. One study that used an ultra-performance liquid chromatography tandem mass spectrometry failed to detect the exact structure corresponding to endogenous ouabain in patients with heart failure and in healthy controls [181] while others reported endogenous ouabain in the blood of various patients [207–210]. Nevertheless, the majority of publications support the presence and functional significance of endogenous ouabain-like compound under physiological and pathological conditions, although its specific molecular identity remains to be validated [211].

Endogenous ouabain concentration is elevated in hypertensive patients

Sub-nanomolar concentrations of endogenous ouabain are detected in the blood of patients with essential hypertension [207–210]. Almost 50% of patients with uncomplicated essential hypertension have been reported to have elevated endogenous ouabain [212]. In accordance with the increased peripheral resistance and normal cardiac output in hypertension, plasma ouabain level correlates positively with elevated peripheral resistance and left ventricular hypertrophy, but not with cardiac output [208,210]. An elevated level of endogenous cardiotonic steroids are also reported in hypertensive patients with volume expansion, for example, kidney and heart failure [213,214], hyperaldosteronism [212], and preeclampsia [215].

Chronic administration of ouabain, leading to an increase of its plasma concentration to the level observed in hypertensive patients, also produces hypertension in rats [61,216,217]. This chronic elevation of blood pressure is

associated with elevated peripheral vascular resistance [61,218]. Accordingly, chronic ouabain treatment induces inward arterial remodeling in the resistance vasculature [219]. Notably, the plasma level of endogenous ouabain is also elevated in several rodent models of hypertension, including reduced renal mass, Milan hypertensive rats, Dahl S rats on high-salt diet and ACTH-induced hypertension [220–223]. Intriguingly, inhibition of endogenous ouabain by systemic administration of the ouabain antagonist, rostafuroxin, or exogenous anti-ouabain antibody lowers blood pressure and even prevents hypertension in rodent hypertension models [224–226]. While anti-ouabain antibodies are thought to scavenge endogenous ouabain [225,226], rostafuroxin is a derivative of another cardiotonic steroid, digitoxigenin, and is suggested to occupy the ouabain-binding site preventing ouabain-induced cSrc activation without affecting the Na,K-ATPase transport [227]. Altogether, it suggests the therapeutic importance of putative endogenous ouabain signaling in hypertension [228].

The Na,K-ATPase may contribute to hypertension via multiple organ systems

Several mechanisms are proposed to link the Na,K-ATPase to hypertension [229]. These include genetically predisposed hyperactivation or ouabain-stimulated overexpression of the Na,K-ATPase in the renotubular epithelium [230,231]. The implication of the renal Na,K-ATPase is supported by observations of elevated endogenous ouabain in the blood of hypertensive animal models [220–223] and patients with volume expansion [213–215]. Moreover, the sympathoexcitatory activity of brain-derived endogenous ouabain is suggested to activate pathways [223] that may contribute to blood pressure elevation by neurogenic potentiation of vascular tone of peripheral resistance arteries [190,191].

Considering that the hemodynamic background of hypertension is an increase in total peripheral resistance, the role of vascular Na,K-ATPase in hypertension is not surprising. As discussed above, the pro-contractile effect of reduced abundancy or activity of the Na,K-ATPase is mainly manifested in small arteries and arterioles [55], which provide a major part of vascular resistance in the circulation [232]. The pro-contractile role of the Na,K-ATPase in these resistance arteries is well-described both *in vivo* [33,233–235] and *in vitro* [11,49,236]. Importantly, sub-micromolar and micromolar concentrations of ouabain itself do not produce significant vasoconstriction but rather potentiate agonist-induced contraction [,49] and myogenic tone [39,165,236,237] of small arteries.

The α_2 isoform is important for the pro-hypertensive action of Na,K-ATPase

To date, only limited information on the hypertension-related proteomic changes in resistance arteries is available, with only one study analyzing the expressional changes in detail. This study did not identify any significant changes in the expression of Na,K-ATPase proteins in mesenteric small arteries from hypertensive rats in comparison with normotensive controls [238]. The α_1 and α_2 isoforms of the Na,K-ATPase are expressed in the arterial wall [40,235,239–242]. However, low plasma concentrations of ouabain are enough to induce hypertension in rodents [216,217], suggesting a primary role of the vascular α_2 isoform. Accordingly, infusion of exogenous ouabain in mice that increases its plasma concentration to approximately 0.1 μM elevates blood pressure and total peripheral resistance in anesthetized mice without any significant effect on cardiac contractility and stroke volume [33]. This is further supported by *ex vivo* studies showing that knockout or down-regulation of the vascular α_2 isoform in rodents leads to potentiation of myogenic contraction [174,235,237,243].

The contribution of the α_2 isoform to vascular agonist-induced contraction is complex, as ouabain has several variables in the acute potentiating effects on the agonist-induced contraction *ex vivo* [39,40,244]. In most *ex vivo* studies, ouabain has been shown to primarily potentiate arterial sensitivity to contractile agonists [11,39,235,245–247], although this effect has not been consistently observed [40,236,242]. Thus, knockout and knockdown of the Na,K-ATPase α_2 isoform potentiates the constriction of neonatal murine aorta in response to thromboxane A_2 analog stimulation [164]. However, the down-regulation of the α_2 isoform in adult rats suppresses the contraction induced by noradrenaline and vasopressin but potentiates pressure-induced myogenic contraction of mesenteric arteries [40]. Further, chronic treatment with low ouabain concentrations does not affect agonist-induced contraction in fourth order mesenteric arteries but potentiates the myogenic tone of these arteries [236]. In contrast, the myogenic and agonist-induced contraction of third-order mesenteric arteries are not affected by chronic ouabain treatment [219]. Altogether, this suggests that the modulatory contribution of α_2 isoform Na,K-ATPase to vascular constriction depends on the vascular bed, arterial size, the contractile stimuli, and whether the effect is chronic or acute.

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Ouabain affects arterial function in a vascular bed-specific manner

Different vascular beds exhibit varying responses to chronic exposure to ouabain in spite of a whole-body increase in blood pressure [242]. Thus, the aorta from chronically ouabain-treated rats is characterized by augmented expression of both α_1 and α_2 isoforms but reduced phenylephrine-induced contraction [242]. Mesenteric superior arteries from the same rats exhibit a similar reduction in contractility but without change in the expression of the Na,K-ATPase α isoforms [242]. In contrast, tail arteries show reduced expression of both Na,K-ATPase isoforms but no difference in the contraction in comparison with normotensive control rats [242]. These results suggest variability of the modulatory effects of ouabain in the vasculature related perhaps to different types and potency of signaling initiated by the Na,K-ATPase, for example, different distribution and subcellular localization of the α isoforms. In fact, the expression density of Na,K-ATPase isoforms varies with the arterial size even within the same vascular bed [49]. While it has been observed that ouabain has a greater effect on vasoconstriction in larger mesenteric artery branches compared with smaller ones, the underlying mechanism has yet to be fully elucidated. However, the arterial caliber-dependent vasocontractile potentiation positively correlates with the expression of the α_2 isoform and the degree of ouabain-induced cSrc kinase phosphorylation [49,236]. These results imply that ouabain in large mesenteric arteries increases vascular smooth muscle cell Ca^{2+} sensitivity in a cSrc-dependent manner (Figure 2) [49,248]. Regardless of the underlying mechanism, the arterial caliber-dependent variability may be important for control of peripheral resistance *in vivo*, where smaller arteries and arterioles provide major resistance under resting conditions, while upstream arteries contribute to hemodynamic resistance during sympathetic excitation [249].

Altering the ouabain-sensitivity of the α_2 isoform confers protection against hypertension

Experimental evidence for the significance of the Na,K-ATPase α_2 isoform in regulating peripheral resistance and blood pressure includes experiments utilizing knock-in mutations of the α isoforms [220,235,250]. Thus, the mutation in the α_2 isoform high-affinity binding site for ouabain [27] modified the normally ouabain-sensitive α_2 isoform to the ouabain-resistant α_2 isoform [251]. This knock-in mutation in mice does not affect the total expression level of vascular Na,K-ATPase α isoforms [250], but it prevents the ouabain-induced and ACTH-induced hypertension in mice [220,235,251]. This was further studied by introducing the mutation in Na,K-ATPase α_1 isoform to increase its ouabain sensitivity [251]. When these two mutations were combined in the knock-in mice, which, in contrast with wild types, had the ouabain-sensitive α_1 isoform and ouabain-insensitive α_2 isoform, they remained resistant to develop hypertension following chronic ouabain treatment [251]. These findings emphasize the crucial role of the ouabain-sensitive α_2 isoform in the development of hypertension [7,252].

Currently there is, however, no generally accepted molecular mechanism that explains how inhibition of the Na,K-ATPase α_2 isoform leads to elevation of blood pressure [45,252]. The situation is further complicated by the fact that not all inhibitors of the Na,K-ATPase have similar effects on blood pressure. Thus, in contrast with ouabain, digoxin does not raise blood pressure and even has antihypertensive action in ouabain-dependent hypertension models [61,216]. Both ouabain and digoxin inhibit the ion pumping action of the Na,K-ATPase *ex vivo* in a specific manner [21,253]. This suggests that the pro-hypertensive action of ouabain is not due to disturbance in intracellular ion homeostasis as it has been validated in *ex vivo* studies [37,38].

Of note, although both ouabain and digoxin are suggested to be specific ligands for the Na,K-ATPase *ex vivo* [21,253], it might be different *in vivo*. Thus, blood plasma immunoglobulin-like molecules were shown to bind ouabain and function as a delivery system for ouabain in the modulation of Na,K-ATPase *in vivo* [254]. Taking into account the difference in molecular charge of ouabain and digoxin [255], it might affect their binding to immunoglobulins *in vivo*. This needs to be tested but different effects of ouabain and digoxin on the arterial contraction *ex vivo* in the experiments with the saline bath solution [11] cannot be explained by immunoglobulin binding. Another concern can be made based on reports from tumor cells showing that in many cardiotonic glycosides, including ouabain and digoxin, binds to the transcriptional regulator steroid receptor co-activators, SRC1 and SRC3 modulating gene expression and tumor cell proliferation [256]. Importantly, the effects of ouabain and digoxin on SRC1 and SRC3 expression in these cells were concentration-dependent and digoxin showed significantly higher potency [256]; however, it has never been shown for other cell types than tumor cells. Further research is needed to test whether the antagonizing effect of digoxin on pro-hypertensive action of ouabain is associated with other than the Na,K-ATPase signaling *ex vivo* and *in vivo*. So although it seems well-documented that the Na,K-ATPase plays a role for the pathophysiology of hypertension, the precise mechanism for this effect is still unclear.

Given the similar putative mechanisms potentially involved in elevating vascular tone discussed in this review for ischemic stroke, migraine with aura, and hypertension, the comorbidity among these disorders may not be surprising.

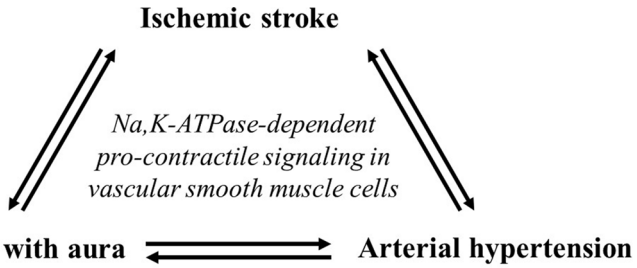

Figure 4. Comorbidities between migrane with aura, ischemic stroke, and hypertension

The hypothetical link between cerebrovascular abnormalities in ischemic stroke, migraine with aura, and hypertension, encompassing Na,K-ATPase-dependent intracellular signaling and Ca^{2+} sensitization in vascular smooth muscle cells.

Migraine with aura significantly increases the risk of ischemic stroke [257–259], while cerebral ischemic events are also proposed to provoke migraine attacks [260,261]. Furthermore, arterial hypertension is a significant risk factor for ischemic stroke [262], and stroke patients with hypertension have a worse outcome compared with normotensive stroke patients [263]. The association between migraine with aura, stroke, and hypertension may involve increased vascular contractility linked to common Ca^{2+} sensitization pathways discussed in this review (Figure 4). Further research is necessary to fully comprehend these comorbidities.

Strengths and limitations

Ouabain is often used in laboratories to inhibit the Na,K-ATPase in tissues of various origins across different species, due to its highly conserved binding site [185,251]. The majority of the studies discussed in this review utilized genetic modifications of the Na,K-ATPase in rodents and/or ouabain to investigate Na,K-ATPase function and its physiological implications. One of the advantages of using ouabain stems from its distinct sensitivity toward the Na,K-ATPase isoforms [27,264]. Particularly, the α_1 isoform in rodents requires significantly higher concentrations of ouabain for effective inhibition compared with the α_2 and α_3 isoforms [29–31]. This disparity allows for pharmacological differentiation and the study of functional roles of these isoforms using ouabain [27,264]. However, it is important to acknowledge the limitations associated with using ouabain to study the Na,K-ATPase. Ouabain has been shown to have effects on cellular processes such as gene expression, protein trafficking, cell adhesion, and proliferation [67,256,265–270]. These effects may be attributed to the changes in ion homeostasis or altered Na,K-ATPase-dependent intracellular signaling but may also be related to off-target binding of ouabain [256]. Unlike other cardiotonic steroids such as digoxin, ouabain is water-soluble, potentially reducing non-specific vehicle effects [271]. It is also worth noting that the concentration and exposure duration of ouabain may influence its effects and may not always reflect physiological conditions accurately.

Conclusions

The vascular Na,K-ATPase is involved in regulating arterial tone, with one mechanism being the modulation of Ca^{2+} sensitivity in arterial smooth muscle cells through cSrc kinase signaling. The Na,K-ATPase-dependent cSrc pathway has been implicated in vascular dysfunction after stroke, as futile recanalization and post-stroke vasospasm. Moreover, reduced expression or pharmacological inhibition of the Na,K-ATPase can lead to cSrc-dependent cerebral hypoperfusion and increased susceptibility to migraine-associated cortical spreading depression. The Ca^{2+} sensitization in smooth muscle modulated by the α_2 isoform may play an important role in the pathophysiology of arterial hypertension, as a pro-hypertensive ouabain-mediated increase in tone of resistance arteries may be facilitated through the amplified cSrc signaling. Targeting the Na,K-ATPase-dependent cSrc signaling pathway may, therefore, provide a potential treatment strategy to prevent excessive smooth muscle cell Ca^{2+} sensitization in multiple vascular disorders.

Data Availability

This is a review article and no original data hence available. The proteomics dataset retrospectively analyzed in this review is openly accessible in the original report (Wen et al., J. Proteome Res., 2019).

Competing Interests

The authors declare that there are no competing interests associated with the manuscript.

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CRediT Author Contribution

Christian Staehr: Conceptualization, Formal analysis, Investigation, Methodology, Writing—original draft, Project administration, Writing—review & editing. **Christian Aalkjaer:** Conceptualization, Supervision, Writing—original draft, Writing—review & editing. **Vladimir V. Matchkov:** Conceptualization, Supervision, Funding acquisition, Investigation, Methodology, Writing—original draft.

Abbreviations

ACTH, adrenocorticotropic hormone; ELISA, enzyme-linked immunosorbent assay; MYPT, myosin phosphatase target subunit 1; NCX, Na,Ca-exchanger.

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