Intracellular pH in the Resistance Vasculature: Regulation and Functional Implications

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Key Words

Endothelial cells · Hypertension · Hypoxia-reoxygenation · Nitric oxide · Resistance arteries · *SLC4A7* · *SLC9A1* · Vascular smooth muscle cells · Vasoconstriction · Vasorelaxation

Abstract

Net acid extrusion from vascular smooth muscle (VSMCs) and endothelial cells (ECs) in the wall of resistance arteries is mediated by the $\text{Na}^+/\text{HCO}_3^-$ cotransporter NBCn1 (*SLC4A7*) and the Na^+/H^+ exchanger NHE1 (*SLC9A1*) and is essential for intracellular pH (pH_i) control. Experimental evidence suggests that the pH_i of VSMCs and ECs modulates both vasoconstrictile and vasodilatory functions in resistance arteries with implications for blood pressure regulation. The connection between disturbed pH_i and altered cardiovascular function has been substantiated by a genome-wide association study showing a link between NBCn1 and human hypertension. On this basis, we here review the current evidence regarding (a) molecular mechanisms involved in pH_i control in VSMCs and ECs of resistance arteries at rest and during contractions, (b) implications of disturbed pH_i for resistance artery function, and (c) involvement of disturbed pH_i in the pathogenesis of vascular disease. The current evidence clearly implies that pH_i of VSMCs and ECs modulates vascular function and suggests that disturbed pH_i either consequent to disturbed regulation or due to metabolic challenges needs to be taken into consideration as a mechanistic component of artery dysfunction and disturbed blood pressure regulation.

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Introduction

Local or systemic deviations in pH may occur under both physiological and pathological conditions. For example, moderate acidification of the local environment has been described during skeletal muscle contractions [1] and high local brain activity [2], while more pronounced local acidification can be seen in malignant tumors [3] and ischemic cardiac tissue [4]. Systemic disturbances in pH are frequently associated with metabolic, renal, gastrointestinal and pulmonary disorders.

Changes in pH affect fundamental cell functions by altering the protonation state of amino acids and hence the charge distribution, conformation and function of proteins. Although most proteins show some degree of sensitivity to changes in pH, certain proteins are much more pH sensitive than others. These particularly pH-sensitive proteins likely serve functions in sensing of pH disturbances and initiation of relevant physiological responses. Examples of highly pH-sensitive proteins include ion channels (e.g. acid-sensing ion channels [5], L-type Ca^{2+} channels [6] and BK channels [7]), G-protein-coupled receptors (e.g. GPR4 [8], OGR1 [8], G2A [9] and TDAG8 [10]) and enzymes (e.g. phosphofructokinase (PFK)-1 [11], NO synthase (NOS) [12, 13] and endothelin-converting enzyme [14]). Although effects of pH on some proteins have been described in detail, the role of altered pH for integrated physiological functions and disease development is only slowly being understood.

It is widely accepted that extracellular pH (pH_o) contributes to the metabolic autoregulation of artery tone and thus participates in adjusting local blood flow to the metabolic demand [15, 16]. In contrast, a comprehensive understanding of how intracellular pH (pH_i) affects vascular function has long been missing. Recently, however, we provided experimental evidence that the Na^+,HCO_3^- cotransporter NBCn1 (*SLC4A7*), by controlling pH_i and hence intracellular enzymatic activities, plays a critical role for maintaining normal function of vascular smooth muscle cells (VSMCs) and endothelial cells (ECs) [13]. In addition to altered artery function, we found that NBCn1 knockout mice have a complex disturbance of blood pressure regulation, being hypertensive at rest but protected against the blood pressure increase induced by N-nitro-L-arginine methyl ester (L-NAME) intake or angiotensin II infusion [13]. The role of NBCn1 in cardiovascular control was recently substantiated by a genome-wide association study demonstrating that the single-nucleotide polymorphism (SNP) rs13082711 located close to the NBCn1 gene (approximately 10 kb 5' of the coding region) is associated with human hypertension [17].

On this basis, it is the aim of the present review to evaluate the current evidence regarding (a) molecular mechanisms involved in pH_i control in VSMCs and ECs of resistance arteries at rest and during contractions, (b) the implications of disturbed pH_i in VSMCs and ECs for resistance artery function, and (c) the involvement of disturbed VSMC and EC pH_i in the pathogenesis of vascular disease.

Mechanisms of pH_i Regulation

The overall mechanisms responsible for pH_i control in VSMCs and ECs are similar to those of most other cell types and involve physicochemical buffering, metabolic acid-base production and membrane acid-base transport [18].

Physicochemical Buffering

Physicochemical buffers are conjugate acid-base pairs important for minimizing changes in pH_i by accepting or donating protons following an acute acid or alkaline load, respectively.

Physicochemical buffering per se does not contribute to determining final steady-state pH_i after recovery from an acid-base perturbation [19] and takes place at such speed that it is usually only apparent when one realizes that an externally induced acute change in pH_i is many orders of magnitude smaller than what one would expect

based on the acid-base load. One exception is the uncatalyzed hydration of CO_2 , which is fairly slow [18]; it has been suggested that part of the recovery seen after an abrupt intracellular alkalization – particularly in the presence of carbonic anhydrase inhibitors – is due to this slow process followed by deprotonation of the created carbonic acid [20].

Mobile intracellular buffers may also play an important role by promoting efficient intracellular diffusion of H^+ equivalents [21] and thus ensure pH_i uniformity, which may at least indirectly enhance membrane acid-base transport and pH_i control. The mobile buffers might also enhance intercellular fluxes of H^+ equivalents through gap junctions [22] and hence permit dissipation of smaller local acid or alkaline loads within a larger cell volume. However, at very low (<6.3) or high (>7.6) pH_i , gap junctions – at least in ventricular cardiomyocytes – have been shown to be almost completely closed [23] thereby protecting surrounding cells from the elevated acid or alkaline load, which could otherwise have deleterious effects.

Metabolic Acid-Base Production

Metabolic processes are an important source of intracellular acid-base equivalents particularly during pathological conditions and may cause pH_i disturbances if membrane transport is not sufficiently activated to dispose of the excess acid load. Interestingly, the interaction between pH_i and the intermediary metabolism is bidirectional, and even a pH_i decrease of 0.2–0.3 units is sufficient to almost completely inhibit the activity of PFK-1 [11] and consequently greatly reduce glycolytic activity [24]. The very strong pH dependency of PFK-1 may therefore serve as a break on glycolytic acid production under conditions of intracellular acidification.

Increased metabolic acid production has been suggested as one possible mechanism contributing to intracellular acid loading during artery contraction [25]. Other explanations, such as enhanced transport of protons over the cell membrane due to increased Ca^{2+} ATPase activity [26, 27], have also been put forward (see the following section and 'Effects of Artery Contraction on pH_i and Regulation of Acid-Base Transport').

Membrane Acid-Base Transport in VSMCs of Resistance Arteries

Intracellular pH of VSMCs is tightly controlled by acid-base transport across the plasma membrane. Under resting physiological conditions, net acid extrusion is mediated exclusively by Na^+,HCO_3^- cotransport and Na^+/H^+ exchange [13, 28, 29], as illustrated in figure 1.

Fig. 1. Schematic representation of the ion transport mediated by the $\text{Na}^+/\text{HCO}_3^-$ cotransporter NBCn1 and the Na^+/H^+ exchanger NHE1. As illustrated, efficient equilibration of the carbonic buffer system means that both transporters mediate net acid extrusion. In most cell types, equilibration of the carbonic buffer system is catalyzed by intra- and extracellular carbonic anhydrases.

Fig. 2. $\text{Na}^+/\text{HCO}_3^-$ cotransport contributes to pH_i regulation in VSMCs of rat mesenteric resistance arteries. **a** The rate of pH_i recovery from an intracellular acid load is much higher in a $\text{CO}_2/\text{HCO}_3^-$ -buffered solution than in a Hepes-buffered solution. Measurements were performed using the pH-sensitive fluorophore BCECF. **b** pH_i recovery from an intracellular acid load in rat mesenteric arteries is paralleled by increased Na^+ influx compared to

baseline. This increase in Na^+ influx during pH_i recovery from intracellular acidification was larger in the presence than in the absence of $\text{CO}_2/\text{HCO}_3^-$. In the experiments, rat mesenteric resistance vessels were investigated in a $\text{CO}_2/\text{HCO}_3^-$ or Hepes-buffered solution and the acid load was induced by incubation in 10 mM NH_4Cl for 20 min prior to the measurement of $^{22}\text{Na}^+$ influx. [This work was originally published in ref. 30; © The Physiological Society.]

The importance of $\text{Na}^+/\text{HCO}_3^-$ cotransport for acid extrusion in VSMCs was originally suggested [30, 31] in isolated rat mesenteric small arteries (fig. 2) but subsequently questioned based on results from guinea pig femoral arteries [32]. Recent studies on mouse arteries confirmed the importance of $\text{Na}^+/\text{HCO}_3^-$ cotransport in VSMCs of mesenteric small arteries [13, 29] and extended these findings to coronary and cerebral small arteries where $\text{Na}^+/\text{HCO}_3^-$ cotransport also plays a prominent role [29] arguing for a general importance in the resistance vasculature. The original studies on rat isolated mesenteric arteries (fig. 2) provided the first functional characterization of an electroneutral and Cl^- -independent $\text{Na}^+/\text{HCO}_3^-$

cotransport mechanism [30, 31]. Later, the electroneutral and Cl^- -independent $\text{Na}^+/\text{HCO}_3^-$ cotransporter NBCn1 was cloned from rat aorta [33], suggesting that also in conduit arteries NBCn1 may play an important role. NBCn1 expression was later demonstrated in VSMCs of mouse mesenteric, coronary and cerebral small arteries [29, 34] (fig. 3), while no expression of other known or putative $\text{Na}^+/\text{HCO}_3^-$ cotransporters of the *SLC4* family was detected [29]. By use of small interfering RNA (siRNA)-mediated knockdown [29] and knockout mice [13], we recently showed that NBCn1 is responsible for the $\text{Na}^+/\text{HCO}_3^-$ cotransport activity in VSMCs of mouse mesenteric arteries during intracellular acidification (fig. 4a, b).

Fig. 3. Na^+ , HCO_3^- cotransporter NBCn1 is strongly expressed in VSMCs and ECs of mouse resistance arteries. **a–e** β -Galactosidase staining of arteries and veins from gene trap mice containing a promoterless LacZ insert under transcriptional control of the NBCn1 promoter. The blue stain is a measure of NBCn1 transcriptional activity. **a** Whole-mount preparation of β -galactosidase-stained mesenteric arteries and veins from homozygous gene trap mice. **b** No staining was seen in mesenteric arteries and veins from wild-type mice. **c** Brain section (8 μm thick) with cerebral arteries and a vein from a homozygous gene trap mouse showing staining of VSMCs and ECs. **d** Kidney section (30 μm thick) with an artery from a homozygous gene trap mouse showing strong staining throughout the VSMC layer (ECs not in focus).

Inset shows kidney artery with ECs in focus. **e** Kidney section (30 μm thick) with an artery from a wild-type mouse; no staining was observed. **f** Immunogold electron microscopy reveals NBCn1 expression in the plasma membrane of VSMCs in the wall of mouse mesenteric arteries. An anti-NH₂-terminal NBCn1 antibody was employed. **g** Immunogold electron microscopy using an anti-NH₂-terminal NBCn1 antibody. NBCn1 expression in the plasma membrane of ECs is seen in the wall of mouse mesenteric arteries. Arrows indicate gold particles associated with the plasma membrane, and arrowheads gold particles associated with intracellular vesicles. (**a–e** [Originally published in ref. 34; © The American Physiological Society]. **f, g** [Originally published in ref. 29 and 13; © The American Heart Association].)

Fig. 4. NBCn1 mediates the Na^+,HCO_3^- cotransport in VSMCs of mouse resistance arteries and is crucial for steady-state pH_i control. **a** After an NH_4^+ prepulse, VSMCs from wild-type mice ($n = 5$) displayed faster pH_i recovery in the presence of CO_2/HCO_3^- than in its nominal absence. **b** After an NH_4^+ prepulse, no difference in pH_i recovery rate was seen between VSMCs from NBCn1 knockout mice ($n = 5$) investigated with or without CO_2/HCO_3^- . At this low pH_i , the contribution of CO_2/HCO_3^- to intracellular buffering capacity is very small compared to the intrinsic buff-

ering capacity. Hence, Na^+,HCO_3^- cotransport in VSMCs is abolished in NBCn1 knockout mice. **c** VSMCs from NBCn1 knockout mice displayed reduced steady-state pH_i compared to wild-type mice ($n = 6$) in the presence but not in the absence of CO_2/HCO_3^- . Comparisons were made by repeated measures two-way ANOVA followed by Bonferroni post hoc tests. * $p < 0.05$. NS = Not significantly different vs. wild type. [This work was originally published in ref. 13; © The American Heart Association.]

Interestingly, while no specific pharmacological tools exist [35], Na^+,HCO_3^- cotransport in VSMCs is inhibited around 75% by 4,4'-diisothiocyanatostilbene-2,2'-disulfonic acid (DIDS) [29, 31]. This is in contrast to most other cell types, where NBCn1 is purportedly insensitive to DIDS [13, 36–38] and different from NBCn1 heterologously expressed in *Xenopus* oocytes, where DIDS inhibits NBCn1 activity only up to 25% [33]. Whether the difference in DIDS sensitivity for NBCn1 expressed in different cell types is due to tissue-specific splice variation (of which multiple are known [39]), posttranslational modifications, protein-protein interactions [for an in-depth discussion, see ref. 35] or other mechanisms is currently unknown.

The ubiquitously expressed Na^+/H^+ exchanger NHE1 (SLC9A1) is the only known plasma membrane Na^+/H^+ exchanger isoform expressed in resistance arteries [40] and cerebrovascular tissue [41]. Using knockout mice, we have very recently shown that NHE1 is responsible for the Na^+/H^+ exchange in VSMCs of isolated mesenteric resistance arteries during intracellular acidification [28] (fig. 5a). These studies were performed on 3- to 4-week-

old mice since the global NHE1 knockout mice die shortly after weaning, presumably due to neurological disorders [42, 43]. The essential role for NHE1 in Na^+/H^+ exchange activity of arterial VSMCs is in agreement with its pharmacological profile being sensitive not only to amiloride but also to low concentrations (1 μM) of cariporide [13].

The relative quantitative importance of Na^+,HCO_3^- cotransport and Na^+/H^+ exchange in VSMCs during intracellular acidification varies substantially between small arteries from mice of different genetic background (e.g. C57BL/6, FVB and NMRI), but taken as a whole, the individual contributions from NBCn1 and NHE1 to net acid extrusion in the pH_i range from 6.5 to 6.8 appear to be of approximately similar magnitude [13, 28, 29, 40], which is also the case in rat mesenteric small arteries [31]. In the near-physiological pH_i range, however, NBCn1 is the dominating mediator of net acid extrusion from resting VSMCs based on the findings that knockout [13] or siRNA-mediated knockdown [29] of NBCn1 or omission of CO_2/HCO_3^- [13, 28, 29] reduce VSMC pH_i up to 0.4 units (fig. 4c). In contrast, knockout of NHE1 only affects

Fig. 5. NHE1 mediates the Na^+/H^+ exchange in VSMCs of mouse resistance arteries. NHE1 is important for steady-state pH_i control only in the absence of CO_2/HCO_3^- . **a** In the absence of CO_2/HCO_3^- , the Na^+ -dependent pH_i recovery was abolished in VSMCs of arteries from NHE1 knockout mice ($n = 5-7$). Experiments were performed in the absence of CO_2/HCO_3^- except for the final part (as indicated). **b** In the absence of CO_2/HCO_3^- , steady-state

pH_i was lower in VSMCs of arteries from NHE1 knockout than wild-type mice. In the presence of CO_2/HCO_3^- , no significant difference in steady-state pH_i was seen between VSMCs of arteries from NHE1 knockout and wild-type mice ($n = 5-8$). Comparisons were made with a two-way ANOVA followed by Bonferroni post hoc tests. *** $p < 0.001$ vs. wild type. [This work was originally published in ref. 28; © Physiological Society.]

steady-state pH_i of VSMCs when investigated in the nominal absence of CO_2/HCO_3^- [28] (fig. 5b). Hence, although NBCn1 and NHE1 perform ostensibly similar transport functions (i.e. net extrusion of 1 H^+ equivalent in exchange for 1 Na^+ ; fig. 1), our findings demonstrate that NBCn1 plays a major role in the control of steady-state pH_i [13, 29] while NHE1 appears to be a major acid extruder at low pH_i but functionally inactive at normal resting pH_i levels [28].

Base extrusion from VSMCs has not been studied to the same extent as acid extrusion. In resistance arteries, there is clear functional evidence for Cl^-/HCO_3^- exchange [31], which appears to play an important role for pH_i recovery from intracellular alkalinization. Based on RNA and protein analyses, AE2 (*SLC4A2*) and AE3 (*SLC4A3*) are expressed in arteries [44], but the precise role of individual Cl^-/HCO_3^- exchangers for VSMC pH_i regulation and artery function remains to be established. Notably, Cl^-/HCO_3^- exchange in VSMCs in addition to a role in pH_i regulation also contributes to maintaining a high intracellular Cl^- concentration [45] and hence to the electro-

chemical gradient favoring Cl^- efflux and membrane depolarization upon opening of Cl^- channels in resting VSMCs [46].

In addition to the dedicated acid-base transporters highlighted above, it should be kept in mind that other membrane transport proteins present in VSMCs also move acid-base equivalents across the plasma membrane. As an example, the transport process mediated by the Ca^{2+} -ATPase includes antiport of H^+ [47], which by acting as counter-ions reduce the electrogenicity and hence the energy requirement of the transport process, but at the same time may contribute to intra- and extracellular pH changes. As described in 'Effects of Artery Contraction on pH_i and Regulation of Acid-Base Transport', H^+ -transport by the plasma membrane Ca^{2+} ATPase has been suggested as a primary mechanism for contraction-induced intracellular acidification of smooth muscle cells [26]. It is also possible that acid-base equivalents such as H^+ or HCO_3^- may permeate ion channels [48, 49], although this has not yet been sufficiently investigated for VSMCs.

It should finally be noted that although the discussion above focuses on ion transport across the plasma membrane, high transport activities for acid-base equivalents are also present in membranes of intracellular organelles, particularly those of lysosomes and mitochondria [50]. In addition to serving important functions for these specialized organelles (e.g. acid-induced activation of intraorganelle enzymes), the accumulation of acid in organelles also removes acid from the cytosol and thereby potentially contributes to control or disturbances of cytosolic pH.

Membrane Acid-Base Transport in ECs of Resistance Arteries

The role of membrane acid-base transport for pH_i control in ECs has been much less studied than in VSMCs. Until recently, our understanding of EC pH_i regulation relied entirely on measurements in cultured cells, but we have now studied the mechanisms of pH_i control in ECs of small isolated arteries using fluorescence confocal microscopy [40]. In our opinion, this is important not only to avoid phenotypic changes during cell culture (see the following section) but also to investigate the relevance of EC pH_i for integrated artery responses such as endothelium-dependent vasorelaxation (see 'Effects of pH_i on Small Artery Function'), which cannot be studied meaningfully in cultured cells.

Previous molecular expression data from artery homogenates suggested that NBCn1 and NHE1 are the only relevant Na^+ -dependent members of the *SLC4* and *SLC9* families expressed in resistance arteries [29, 40]. Evidence for NBCn1 expression in ECs of small arteries has also been provided [13, 34, 51] (fig. 3); recently, we showed functionally that NBCn1 and NHE1 are responsible for the Na^+ -dependent acid extrusion from ECs [13, 28]. In congruence with our findings in VSMCs, we found that while both NBCn1 and NHE1 contribute to acid extrusion during intracellular acidification, only NBCn1 appears to be active in the near-physiological pH_i range and consequently important for steady-state pH_i control in ECs under resting physiological conditions [13, 28]. In contrast to VSMCs, the transport by NBCn1 in ECs was unaffected by 200 μM DIDS [13]. The reason for this difference in pharmacological sensitivity between NBCn1 expressed in these closely appositioned cell types is currently unknown. For a more elaborate discussion of the effects of DIDS, see Boedtker et al. [35]. As expected, and similar to VSMCs, the Na^+/H^+ exchange in ECs was inhibited by amiloride or low concentrations (1 μM) of the NHE1-selective inhibitor cariporide [40].

Membrane Acid-Base Transport in Other Blood Vessels and Cultured Vascular Cells

The transport characteristics described above are based on experiments with mesenteric, coronary and cerebral small arteries with an internal diameter between 150 and 250 μm [29]. Similarly sized arteries have been shown to contribute significantly to vascular resistance [52] and hence to the regulation of local blood flow and control of systemic blood pressure. It is likely, however, that the findings regarding molecular mechanisms of pH_i control are also applicable to other arterial beds and differently sized arteries. Hence, we have found that NBCn1 is responsible for the $\text{Na}^+/\text{HCO}_3^-$ cotransport in conduit arteries [unpubl. observation], and NBCn1 expression has been detected in renal arteries [34] (fig. 3d), heart capillaries [51] and also in veins [34] (fig. 3a, c). Despite this, we cannot of course completely exclude that other transport mechanisms than $\text{Na}^+/\text{HCO}_3^-$ cotransport and Na^+/H^+ exchange or different NBC or NHE isoforms may play a role in blood vessels of different size or origin or under different physiological or pathological conditions.

It is well known that cells in culture differ from native cells in a number of ways. It is not surprising therefore that although Na^+ -dependent HCO_3^- transport has been described in cultured VSMC- and EC-like cells [53–56], the characteristics of this transport differ somewhat from the transport observed in isolated arteries (see the two preceding sections) and has for instance been suggested to be at least partly coupled to net Cl^- transport [54–56]. These differences in transport characteristics between cultured cells and freshly isolated tissue preparations are most easily explained by a change in protein expression pattern during the culture process. This explanation seems further supported by the finding that in contrast to isolated arteries, cultured ECs from brain microvessels [57] and cultured rat aortic smooth muscle cells (A7r5 [unpubl. observation]) express transcripts for a large number of different Na^+ -dependent HCO_3^- transporters.

Another important example of culture-induced changes relating to artery function is the shift from a contractile to a synthetic VSMC phenotype [58]. The loss of contractile function seen during culture obviously renders cultured VSMCs less representative of native arterial VSMCs and unsuited for studies regarding vasomotor characteristics. Since many of the culture-induced changes also take place in response to vascular injury and are prominent in atherosclerotic and restenotic lesions [59], studies on cultured VSMCs may, however, provide important insights into mechanisms involved in the de-

velopment and progression of these prevalent vascular conditions. Considering the importance of acid-base transport for altering fundamental cell functions (e.g. cell proliferation and migration) implicated in changes of artery morphology [28, 60, 61] (see also 'Hypertension'), it may moreover be very relevant to study pH_i -regulatory pathways in VSMCs from arteries with atherosclerotic or restenotic lesions.

Effects of pH_o on pH_i

In the vascular wall, changes in pH_o are followed by changes in pH_i in the same direction, and a new steady-state pH_i is typically reached within a couple of minutes [62, 63]. Although reports vary, there is general consensus that the change in steady-state pH_i is between 50 and 75% of the imposed change in pH_o [63–65]. The magnitude of change in pH_i depends, however, on whether the extracellular acid-base disturbance is respiratory (i.e. hyper- or hypocapnic) or metabolic (i.e. normocapnic) in nature, with pH_i being more sensitive to respiratory than metabolic disturbances [62, 64].

The change in pH_i following a change in pH_o purportedly results from altered movement of acid-base equivalents across the plasma membrane. This may be explained by changes in fluxes of small ionic species such as H^+ , OH^- or HCO_3^- through ion channels, a mechanism supported for instance by the finding that several Cl^- channels are also permeable to HCO_3^- [48, 49] (see also 'Membrane Acid-Base Transport in VSMCs of Resistance Arteries'). In addition, uncharged forms of acid-base pairs or related species (e.g. monocarboxylic acids, CO_2 or NH_3 ; see also 'Effects of pH_i on Small Artery Function') move rapidly across cell membranes by passive diffusion through the lipid bilayer or through gas channels [66–71], whereas charged species such as metabolic monocarboxylates can move via specific transporters expressed in the VSMC plasma membrane [72]. Furthermore, it is well established that the Na^+/H^+ exchangers are inhibited by extracellular acidosis [73, 74], which will further contribute to the intracellular buildup of protons. $\text{Na}^+/\text{HCO}_3^-$ cotransport also appears to be inhibited by acidic extracellular conditions, but whereas maximal inhibition of Na^+/H^+ exchange has been reported around pH_o 6.8, maximal inhibition of $\text{Na}^+/\text{HCO}_3^-$ cotransport requires pH_o levels below 6.3 [75]. Further investigations are needed to clarify the relative contributions from each of these mechanisms in the vascular wall and determine their functional impact in ECs and VSMCs.

Effects of pH_i on Small Artery Function

A large number of studies have investigated the response of arteries to acute intracellular acid-base disturbances induced by addition and subsequent washout of weak acids or bases such as NH_4^+ , acetate, lactate, pyruvate or butyrate [66–69], which in aqueous solution coexist with their conjugate acid or base. These maneuvers typically cause pH_i changes of large magnitude (often around 0.5 units) and take place within a few tens of seconds [29]. The pH_i changes observed are explained by the relative permeability of the plasma membrane to the acid and its conjugate base. During a so-called NH_4^+ prepulse [18], the pH_i responses are: (a) an abrupt intracellular alkalization upon extracellular addition of NH_4Cl due to fast influx of NH_3 through the lipid bilayer and potentially via gas channels, (b) a gradual recovery of pH_i due to slower influx of NH_4^+ through K^+ conductances and base extrusion by the anion exchangers, and (c) an abrupt intracellular acidification upon washout of extracellular NH_4Cl as NH_3 quickly leaves the cells and H^+ is liberated from intracellular NH_4^+ . The vast majority of studies from resistance arteries report that acute intracellular acidification induced by an NH_4^+ prepulse or by an acute hypercapnic challenge causes vasoconstriction [30, 76–79] due to an increase in intracellular $[\text{Ca}^{2+}]$ in the VSMCs [80, 81]. There are only few examples of studies suggesting vasodilatation in response to acute intracellular acidification [63]. The mechanisms linking acute changes in VSMC pH_i to changes in $[\text{Ca}^{2+}]_i$ are not completely clear, but effects on membrane potential [7, 81, 82], transport of Ca^{2+} across the plasmalemma or the sarcoplasmic reticulum membrane [79, 83–85] and competition between H^+ and cytosolic Ca^{2+} for buffer binding [80, 86] have all been suggested.

Studies investigating how sustained changes in pH_i modify contractile responses of small arteries have previously been limited by the difficulty of experimentally inducing prolonged intracellular acidification without changing extracellular conditions. Using knockout mice for acid-base transporters, we have, however, recently been able to circumvent these difficulties [13, 28]. The effects of sustained changes in pH_i on arterial tone regulation appear more subtle than those induced for instance by washout of NH_4Cl ; but as detailed below (both following sections), this may in part be due to opposing effects of low pH_i in the VSMCs and ECs [13, 28], i.e. low VSMC pH_i tends to reduce VSMC contractility while low EC pH_i tends to reduce NO-mediated vasodilation and hence increase contractility. The effects of sustained changes in

Fig. 6. Diagrammatic representation of the pathways affected by sustained changes in pH_i in the arterial wall. As illustrated in figure 1, NBCn1 contributes to pH_i control by transporting HCO_3^- , which in turn consumes cytosolic H^+ . ACh = Acetylcholine; Ang-II = angiotensin II; CaM = calmodulin; DAG = diacylglycerol; eNOS = endothelial NOS; IP_3 = inositol 1,4,5-trisphosphate; MLC-P = phosphorylated MLC; MLCK = MLC kinase; MLCP = MLC phosphatase; MLCP-P = phosphorylated (inactive) MLC phosphatase; NA = noradrenaline; PIP_2 = phosphatidylinositol-4,5-bisphosphate; V_m = membrane potential. The plasma membrane ion channel illustrates Ca^{2+} entry through receptor-activated cation channels and voltage-sensitive Ca^{2+} channels. The ion channel in the sarcoplasmic reticulum represents Ca^{2+} release through IP_3 receptors. As illustrated, there is evidence for a direct inhibitory effect of low pH on rho kinase activity [13], but the inhibition of rho-kinase-mediated signaling by pH_i may also in part be secondary to effects further upstream in the signaling cascade.

logical rho kinase inhibition (using e.g. Y-27632 or fasudil) on resistance artery function is dramatic and complete inhibition in mesenteric small arteries reduces noradrenaline-sensitivity ($-\log\text{EC}_{50}$) by a half log unit and maximal active tension development by approximately 50% [13]. The isolated rho kinase *in vitro* has a smaller intrinsic pH sensitivity [13] than some of the most pH -sensitive enzymes described to date (e.g. the NOS [12] and PFK1 [11]). However, since no more than 10% pharmacological inhibition of the rho kinase is required to significantly reduce noradrenaline sensitivity with respect to tension development [13], even a moderate intrinsic pH sensitivity is likely to have important biological consequences. Notably, we have shown that pharmacological and pH_i -mediated rho kinase inhibition of similar magnitude produces approximately equivalent changes in artery contractions [13] suggesting that the intrinsic pH sensitivity of the rho kinase plays a significant role for pH_i -mediated effects on artery function. Reduced rho kinase activity at low pH_i was further confirmed by our finding that phosphorylation of the myosin light chain (MLC) phosphatase targeting subunit (MYPT) at Thr^{850} was reduced in arteries from NBCn1-knockout mice [13]. Consistent with effects of pH_i on rho-

pH_i on vascular function are summarized in figure 6 and addressed in further detail below.

Effects of Sustained Intracellular Acidification on VSMC Function

As mentioned above, acute changes in pH_i have prominent effects on the intracellular Ca^{2+} concentration in VSMCs [80, 81]. In contrast, Ca^{2+} handling in VSMCs does not appear to be affected during prolonged intracellular acidification [13, 29]. Interestingly, changes in pH_i have been suggested to influence VSMC Ca^{2+} sensitivity [13, 28, 29, 87, 88], although early studies employing chemical permeabilization were in conflict as to whether the Ca^{2+} sensitivity was increased or reduced at low pH_i [87, 88]. Using knockout mice for NBCn1 and NHE1, we recently investigated the effects of sustained intracellular acidification on VSMC function under physiological extracellular conditions [13, 28]. After pharmacological inhibition of endothelium-dependent vasorelaxation in arteries from both mouse models, we found that intracellular acidification reduced VSMC Ca^{2+} sensitivity and showed moreover that this effect was abolished following rho kinase inhibition [13, 28]. The influence of pharmaco-

logical rho kinase inhibition (using e.g. Y-27632 or fasudil) on resistance artery function is dramatic and complete inhibition in mesenteric small arteries reduces noradrenaline-sensitivity ($-\log\text{EC}_{50}$) by a half log unit and maximal active tension development by approximately 50% [13]. The isolated rho kinase *in vitro* has a smaller intrinsic pH sensitivity [13] than some of the most pH -sensitive enzymes described to date (e.g. the NOS [12] and PFK1 [11]). However, since no more than 10% pharmacological inhibition of the rho kinase is required to significantly reduce noradrenaline sensitivity with respect to tension development [13], even a moderate intrinsic pH sensitivity is likely to have important biological consequences. Notably, we have shown that pharmacological and pH_i -mediated rho kinase inhibition of similar magnitude produces approximately equivalent changes in artery contractions [13] suggesting that the intrinsic pH sensitivity of the rho kinase plays a significant role for pH_i -mediated effects on artery function. Reduced rho kinase activity at low pH_i was further confirmed by our finding that phosphorylation of the myosin light chain (MLC) phosphatase targeting subunit (MYPT) at Thr^{850} was reduced in arteries from NBCn1-knockout mice [13]. Consistent with effects of pH_i on rho-

Fig. 7. Blood pressure effects of disturbed pH_i regulation. NBCn1 knockout mice are mildly hypertensive at rest, display attenuated blood pressure responses to NOS and rho kinase inhibition and are resistant to developing hypertension to angiotensin II. **a** Resting mean arterial blood pressure (MAP) was higher in NBCn1 knockout mice compared to wild-type mice while resting heart rate (HR) was not significantly different. The measurements were performed by radiotelemetry ($n = 12\text{--}14$) and similar results were obtained by tail cuff measurements (not shown). **b** The increase in mean arterial blood pressure (ΔMAP) upon treatment with L-NAME in the drinking water was attenuated in NBCn1 knockout mice compared to wild-type mice ($n = 12\text{--}14$). L-NAME intake relative to body weight was not different between NBCn1 knock-

out and wild-type mice (not shown). **c** The change in mean arterial blood pressure (ΔMAP) following injection of Y-27632 (10 mg/kg body weight i.p.) was smaller in NBCn1 knockout compared to wild-type mice. The star denotes significant interaction (i.e. the effect of Y-27632 was significantly different between NBCn1 knockout and wild-type mice; comparison was made with repeated-measure two-way ANOVA). **d** Mean arterial blood pressure before and during continuous angiotensin II infusion by an osmotic minipump in NBCn1 knockout and wild-type mice ($n = 7\text{--}8$). The increase in mean arterial blood pressure during angiotensin II infusion was reduced in NBCn1 knockout compared to wild-type mice. [This work was originally published in ref. 13; © The American Heart Association.]

kinase-dependent signaling in vivo, the blood pressure responses to pharmacological inhibition with Y-27632 or stimulation with angiotensin II were attenuated in NBCn1 knockout compared to wild-type mice [13] (fig. 7c, d; see also 'Hypertension').

In principle, effects of pH_i on VSMC Ca^{2+} sensitivity could be due not only to signaling cascades modulating MLC phosphatase activity but also secondary to effects on the MLC kinase. This, however, seems unlikely because (a) the difference in VSMC Ca^{2+} sensitivity in that case would not be expected to be sensitive to rho kinase inhibition, (b) experimental evidence suggests that the

MLC kinase displays almost constant catalytic activity in the broad pH spectrum between 6.5 and 9.0 [89], and (c) binding of Ca^{2+} to calmodulin and the interaction of the Ca^{2+} -calmodulin complex with its target proteins including the MLCK are either unaffected or even slightly enhanced at low pH [90, 91].

In studies based on genetically modified animals, it is imperative to consider whether the observed phenotypic changes are directly caused by the altered expression of the protein in question or secondary for instance to compensatory up- or downregulation of other proteins. The causative role of pH_i in our experiments was strongly sup-

ported by the comparable effects of NBCn1 and NHE1 knockout observed under conditions of intracellular acidification [13, 28]. The similar effects of low pH_i observed in arteries from NBCn1 and NHE1 knockout mice under in vitro conditions furthermore suggest that acute rather than chronic (e.g. developmental) effects of pH_i are responsible for the phenotype since intracellular acidification under in vivo circumstances (i.e. in the presence of $\text{CO}_2/\text{HCO}_3^-$) is expected in the NBCn1 but not in the NHE1 knockout mice [13, 28] (fig. 4c, 5b; see also 'Membrane Acid-Base Transport in VSMCs of Resistance Arteries' and 'Membrane Acid-Base Transport in ECs of Resistance Arteries'). In addition, the causative role and acute effect of pH_i was supported by the finding that the lower VSMC Ca^{2+} sensitivity in arteries from NBCn1 knockout mice was evident only in the presence of $\text{CO}_2/\text{HCO}_3^-$ when pH_i is reduced [13]. In the absence of $\text{CO}_2/\text{HCO}_3^-$, no difference in pH_i was present and no difference in Ca^{2+} sensitivity was observed [13]. Conversely, effects of NHE1 knockout on both pH_i and Ca^{2+} sensitivity were seen only in the absence of $\text{CO}_2/\text{HCO}_3^-$ [28].

Effects of Sustained Intracellular Acidification on EC Function

The vascular endothelium of resistance arteries is responsible for initiating a large number of signaling cascades modulating active tension development in the surrounding VSMCs. Both vasoconstrictile (e.g. endothelin and thromboxane) and vasodilatory factors or pathways (e.g. NO, prostacyclin and endothelium-derived hyperpolarizing factor) can be released or initiated from the endothelium [92]. In most cases, however, activation of the endothelium with agonists such as acetylcholine or bradykinin or mechanical stimulation by increased shear stress initiate a predominantly vasodilatory response [13, 93].

In light of the high secretory activity of the vascular endothelium and the potential of pH to alter enzymatic activity, it is not surprising that changes in pH_i modify EC function. Using knockout mice for NBCn1 and NHE1, we have shown that the vasodilatory response to acetylcholine stimulation is greatly attenuated at low pH_i [13, 28]. Under $\text{CO}_2/\text{HCO}_3^-$ -buffered conditions where the ECs in arteries from NBCn1 knockout mice are acidified around 0.25 pH units compared to arteries from wild-type mice, endothelium-dependent vasorelaxation was approximately 30% reduced [13]. This difference in vasodilatory response between arteries from NBCn1 knockout and wild-type mice was completely abolished after L-NAME preincubation suggesting an effect on NO-dependent signaling [13]. In principle, reduced NO-mediated signaling

may arise due to inhibited NO synthesis, enhanced NO degradation or reduced effect of NO on the VSMCs. The response to the exogenous NO donor S-nitroso-N-acetylpenicillamine was similar in arteries from NBCn1 knockout and wild-type mice and preincubation with the superoxide scavengers tempol or polyethylene glycol superoxide dismutase did not rescue the vasodilatory response of arteries from NBCn1 knockout mice suggesting an effect on NO synthesis [13]. In agreement, intraluminal concentrations of NO (measured with NO-sensitive electrodes) during acetylcholine stimulation were reduced in mesenteric arteries from NBCn1 knockout compared to wild-type mice, and NOS activity (measured as L-NAME-sensitive arginine to citrulline conversion) in intact aortic segments was attenuated [13]. In support of pH_i being the causative link between NBCn1 knockout and reduced endothelium-dependent vasorelaxation, there was no difference in vasodilatory function between arteries from NBCn1 knockout and wild-type mice when investigated in the absence of $\text{CO}_2/\text{HCO}_3^-$ where pH_i is similar in the two groups [13]. Further substantiating this observation, we recently showed that EC pH_i in arteries from NHE1 knockout mice was reduced around 0.5 units when investigated in the absence of $\text{CO}_2/\text{HCO}_3^-$ and under these conditions L-NAME-sensitive vasodilatory responses to acetylcholine were almost completely abolished [28]. In contrast, in the presence of $\text{CO}_2/\text{HCO}_3^-$, both EC pH_i and the vasodilatory response to acetylcholine were very similar between arteries from NHE1 knockout and wild-type mice [28]. In arteries from both NBCn1 and NHE1 knockout mice, endothelial Ca^{2+} handling appeared unaffected by low pH_i [13, 28]. In congruence with our findings in isolated arteries, earlier studies have shown that the isolated NOS when investigated in vitro has a bell-shaped pH dependency with maximum activity around pH 7.5 and substantially reduced activities at both lower and higher pH values even within the physiological pH_i range [12]. Supporting the conclusion that NO production is inhibited at low pH_i due to the intrinsic pH sensitivity of the endothelial NOS, we found no difference in the level of total or Ser¹¹⁷⁷ phosphorylated NOS expression between arteries from NBCn1 knockout and wild-type mice [13].

The effects of NBCn1 knockout on NO-mediated responses in isolated resistance arteries were paralleled by an increased resting blood pressure (fig. 7a) and an almost completely abolished blood pressure increase to L-NAME treatment in NBCn1 knockout compared to wild-type mice [13] (fig. 7b; see also 'Hypertension') suggesting that the pH_i -mediated inhibition of NOS activity is also important in vivo.

Effects of Artery Contraction on pH_i and Regulation of Acid-Base Transport

Several studies have investigated the effect of vasoconstriction on VSMC pH_i . When investigated in the presence of $\text{CO}_2/\text{HCO}_3^-$, acute contractions induced by noradrenaline [29], angiotensin II [27], Ca^{2+} ionophores [27, 29] or membrane depolarization with elevated extracellular concentrations of K^+ [25] have been reported to cause intracellular acidification within the first few minutes. The reason for this acidification has not been conclusively determined, but the acidification has been shown to be secondary to the rise in intracellular Ca^{2+} concentration, since it is inhibited by intracellular loading with the Ca^{2+} chelator BAPTA [27]. Intracellular acid loading via membrane transport has been suggested as a mechanism for the contraction-induced intracellular acidification: the Ca^{2+} -ATPase transports Ca^{2+} across cell membranes in exchange for H^+ , and since it does so at a higher rate during contractions, it may contribute to the intracellular acidification [26, 27]. Supporting this notion, extracellular alkalinization has been reported during contraction-induced intracellular acidifications [26]. The initial acidification described above has in several studies been reported to be followed by a slower recovery of pH_i [29], which is dependent on $\text{CO}_2/\text{HCO}_3^-$ [25, 29] and inhibited by DIDS [25]. The acidification has furthermore been shown to be augmented in the absence of extracellular Na^+ [25]. Taken together, these findings strongly suggest that $\text{Na}^+/\text{HCO}_3^-$ cotransport in VSMCs is activated during contractions of rat mesenteric arteries and plays an important role for pH_i control under these conditions. Activation of Na^+ -dependent HCO_3^- transport by agonist stimulation has also been reported in other cell types [94, 95], but the intracellular signaling mechanisms still need to be investigated.

Involvement of pH_i Disturbances in Vascular Disease

As outlined in 'Effects of pH_i on Small Artery Function', changes in EC and VSMC pH_i alter both vasodilatory and vasocontractile functions. It is therefore conceivable that dysregulation of pH_i in ECs or VSMCs contributes to disease development in conditions involving vascular dysfunction. In addition, particularly NHE1 may have cellular effects, which act not only via changes in pH but may also rely on interactions with extracellular matrix components, the cytoskeleton or intracellular signaling pathways [96, 97]. The pH_i -independent effects

have been shown to be important for cell migration and proliferation [96, 97] and hence may be of consequence for arterial structure development [28].

The hypothesis that acid-base transport and pH_i contribute to disease development or associated complications has fostered a number of studies proposing among others that pH_i disturbances are associated with vascular manifestations of hypertension and development of endothelial dysfunction following hypoxia-reoxygenation events. The current evidence for involvement of NBCn1 or NHE1 in these conditions is discussed below.

Hypertension

Considering the effects of VSMC and EC pH_i on both vasocontractile and vasodilatory functions of resistance arteries and hence potentially on peripheral vascular resistance, it is obvious to ask whether pH_i and altered function of membrane acid-base transporters contribute to blood pressure disturbances. This question was recently addressed by a genome-wide association study showing that the SNP rs13082711 close to the NBCn1 gene (approximately 10 kb 5' of the coding region) is associated with hypertension in humans [17]. Although the effect size was rather small (around 0.3 mm Hg), the finding is consistent with NBCn1 being of major importance for vascular function and having therapeutic potential in the treatment of vascular disease. It is important to recognize that the effect size of a given SNP does not necessarily correlate well with the therapeutic potential of that target. For instance, SNPs located in the gene for HMG-CoA (3-hydroxy-3-methyl-glutaryl-coenzyme A) reductase have small effect sizes explaining only a 5% difference in LDL cholesterol [98, 99]; yet, inhibition of the HMG-CoA reductase with statins reduces LDL cholesterol levels substantially (30–40% [100]) and are used extensively to protect against cardiovascular events. This difference between genetic and pharmacological effects is likely explained by SNPs altering the activity or expression of a given target only slightly while pharmacological intervention may allow for an almost complete inhibition [98]. At present, it is unclear whether the rs13082711 SNP alters NBCn1 expression or is in disequilibrium with SNPs inside the NBCn1 coding region, which may confer a change in transport activity. Experiments to address potential effects of the rs13082711 SNP on NBCn1 function and expression would provide important information regarding the role of NBCn1 and pH_i for human cardiovascular function in vivo.

The animal studies described above ('Effects of Sustained Intracellular Acidification on VSMC Function'

and 'Effects of Sustained Intracellular Acidification on EC Function') imply that NBCn1 plays a considerable role in vivo: global NBCn1 knockout mice have a complicated blood pressure disturbance being mildly hypertensive at rest but protected against the blood pressure increase induced by NOS inhibition (with L-NAME) or angiotensin II infusion [13] (fig. 7). Apparently, the balance between activities in the NO- and rho-kinase-dependent signaling cascades under a given condition determines whether the NBCn1 knockout mice have higher or lower blood pressure relative to the wild-type mice. The current evidence strongly implies that NBCn1 has potential for modulating artery function and blood pressure regulation in mice. Although genetic evidence suggests that similar mechanisms are at play in humans, further verification is required to determine the potential pathophysiological role and therapeutic potential of NBCn1 in human vascular disease and blood pressure dysregulation.

Studies have shown that Na^+/H^+ exchange activity is increased in erythrocytes and platelets from up to 50% of humans with primary hypertension [101], whereas linkage analyses and sequencing data have provided no evidence for mutations in NHE1 associated with hypertension [102–104]. Increased Na^+/H^+ exchange activity has also been described in arteries from spontaneously hypertensive rats (SHR) compared to Wistar-Kyoto (WKY) control rats [105–107] resulting in an increased VSMC steady-state pH_i and a faster recovery from intracellular acidification in the absence of $\text{CO}_2/\text{HCO}_3^-$ [106]. The majority of data suggests no difference in total NHE1 protein or mRNA level between VSMCs from SHR and WKY rats [108–112], although at least one study employing cultured VSMCs has reported higher NHE1 abundance in SHR compared to WKY [113]. No mutations within the coding region of NHE1 have been found in SHR [107] and the mechanistic background for the increased Na^+/H^+ exchange activity therefore remains undetermined, although effects on trafficking, protein stability, posttranslational modifications [35] or the activity of signaling cascades modulating NHE1 activity [35] could be involved. In line with the above description that NHE1 is an important acid extruder at low pH_i but functionally inactive in the near-physiological range [13, 28] (see also 'Membrane Acid-Base Transport in VSMCs of Resistance Arteries' and 'Membrane Acid-Base Transport in ECs of Resistance Arteries'), no difference in steady-state pH_i or recovery rate after an intracellular acid load was seen between arteries from adult SHR and WKY rats in the presence of $\text{CO}_2/\text{HCO}_3^-$ [105, 106]. Interestingly, evidence has been provided for a more alkaline pH_i in arteries from young (5-week-old) SHR compared to WKY

rats [105]. In vivo, one would not expect any difference in bulk VSMC pH_i between adult SHR and WKY rats and it seems unlikely that acute effects of pH_i are directly responsible for the hypertensive phenotype observed. Nonetheless, NHE1 per se could be important since it has been shown to have transport-independent effects on phenomena such as cell migration and proliferation [96, 97]. It should also be noted that the alkaline pH_i measured in the young SHR coincides temporally with the development of hypertension and inward arterial remodeling [105], which may provide a long-term structural effect even after the pH_i difference disappears. Although differences in bulk VSMC pH_i are not expected under physiological conditions, it is also possible that pH differences in subcellular domains or restricted extracellular compartments are present and play a role.

Transgenic mouse models have also provided insights relevant to the question of NHE1 in hypertension. Mice overexpressing NHE1 have salt-sensitive hypertension [114], while NHE1 knockout mice are hypotensive and display altered artery structure [28]. In line with a role of NHE1 for cell proliferation and migration, our recent findings suggest that NHE1 is important for normal development of artery morphology in the systemic circulation since arteries from NHE1 knockout mice have a reduced media to lumen ratio [28]. It should be noted, however, that NHE1 knockout mice are hypotensive [28] and whether the thinning of the arterial walls is a consequence of or contributes to the low blood pressure requires further investigation.

While the discussion above has focused on the role of pH_i and membrane acid-base transport in arteries from the systemic circulation, recent evidence suggests that intracellular acid-base homeostasis also plays a very significant role in the pulmonary circulation. Knockout mice for NHE1 have been shown to be unsusceptible to developing pulmonary hypertension and pulmonary artery remodeling [60]. In accordance with a role for NHE1 in pulmonary artery remodeling, siRNA-mediated knockdown of NHE1 was subsequently shown to inhibit proliferation, migration and hypertrophy of VSMC-derived cells [61].

Hypoxia-Reoxygenation

Reduced blood perfusion typically results in acid-base disturbances, and a number of studies have suggested that acid-base transport plays a role for the outcome of ischemic disease. As such, hypoxia-reoxygenation causes myocardial damage, which can be reduced by prior inhibition of Na^+/H^+ exchange [115–117]. Similarly, endothe-

lial dysfunction can be caused by hypoxia-reoxygenation [118–120] and beneficial effects of specific Na^+/H^+ exchange inhibitors have been suggested [120, 121]. While these findings support that Na^+/H^+ exchange is involved in the pathogenesis of ischemic cell injury, the relative contribution of Na^+/H^+ exchange inhibition in cardiomyocytes, ECs and potentially other cell types to alleviate development of myocardial dysfunction still requires further investigation. Mechanistically, the Na^+ -loading capacity of the Na^+/H^+ exchangers may, by affecting $\text{Na}^+/\text{Ca}^{2+}$ exchange activity, result in intracellular Ca^{2+} overload and consequent cell injury. The results are, however, not unambiguous and inhibitors of Na^+/H^+ exchange have been suggested to defend cardiomyocytes against reoxygenation damage owing to a reduced Ca^{2+} overload [122] or independently of Ca^{2+} overload [123].

We recently showed that H_2O_2 inhibits Na^+/H^+ exchange in arterial ECs in situ [40] and considering the increased amounts of reactive oxygen species present during ischemia-reperfusion events [124], we suggested that H_2O_2 -mediated NHE1 inhibition might act as a cellular defense mechanism to reduce cellular Na^+ uptake, intracellular Ca^{2+} overload and cell damage [40]. Interestingly, we furthermore showed that insulin – which has been shown to protect against ischemia-reperfusion injury [125] – stimulates intracellular H_2O_2 accumulation in the vascular wall and inhibits Na^+/H^+ exchange activity [40]. The effects of insulin were prominent in ECs but evident in VSMCs primarily at supraphysiological concentrations [40]. This could indicate that the ECs are the source of the H_2O_2 production which would be consistent with the lack of an inhibitory effect of insulin on Na^+/H^+ exchange activity in primary monocultures of canine VSMCs [126], although the apparent difference could also be explained by much lower concentrations of insulin used in the cell culture experiments.

To exploit the potential beneficial effects of NHE1 inhibition on the outcome of ischemic heart disease, clinical trials using the Na^+/H^+ exchange inhibitors cariporide or eniporide to treat cardiac disease have been performed [127, 128], but even though positive treatment effects were seen in some patient subgroups, the development of neurological side effects has precluded wider clinical use.

Considering the ostensibly similar transport functions of NHE1 and NBCn1 [35], it seems likely that NBCn1 is also involved in the development of cellular dysfunction after hypoxia-reoxygenation. In fact, in the arterial wall, $\text{Na}^+/\text{HCO}_3^-$ cotransport could be expected to contribute more to cellular Na^+ uptake than NHE1 since (a) at resting pH_i values, NBCn1 is more active than

NHE1 while both transporters are activated by intracellular acidification [13, 28, 29], (b) compared to NHE1 activity, which is strongly inhibited by extracellular acidification, $\text{Na}^+/\text{HCO}_3^-$ cotransport activity is much less affected [75], and (c) during hypoxia-reoxygenation events, local concentrations of reactive oxygen species rise [124], which inhibits NHE1 activity but has no effect on NBCn1 activity [40]. Although the contribution of $\text{Na}^+/\text{HCO}_3^-$ cotransport to myocardial damage following ischemia-reperfusion has not yet been conclusively determined, in one study it has at least been shown that combined inhibition of Na^+/H^+ exchange (using cariporide) and $\text{Na}^+/\text{HCO}_3^-$ cotransport (using DIDS) is necessary to protect against the development of reoxygenation-induced hypercontracture while either inhibitor alone is insufficient [129]. Unfortunately, no pharmacological $\text{Na}^+/\text{HCO}_3^-$ cotransport inhibitor with sufficient specificity for in vivo administration has been described up to now [35] limiting both experimental investigations of potential benefits of $\text{Na}^+/\text{HCO}_3^-$ cotransport inhibition and eventual clinical applications.

Conclusion and Perspectives

Increasing experimental evidence suggests that pH_i of VSMCs and ECs modulate vascular function. Acute intracellular acidification has been consistently shown to cause VSMC contraction due to an increase in intracellular $[\text{Ca}^{2+}]$. Longer-term pH_i regulation is governed by the $\text{Na}^+/\text{HCO}_3^-$ cotransporter NBCn1 and the Na^+/H^+ exchanger NHE1, which are responsible for net cellular acid extrusion (fig. 1) and play significant roles for maintaining normal artery structure and function. During sustained intracellular acidification, vasoconstrictile as well as vasodilatory functions are inhibited. The link between sustained disturbances in pH_i and the development of vascular dysfunction involves pH_i -mediated changes in intracellular enzymatic activity with consequences for NO-mediated and rho-kinase-dependent signaling (fig. 6). Most cell functions depend upon efficient catalysis of key biochemical reactions, and it is consequently likely that many other aspects of cell function than vasomotor function are also affected by disturbed pH_i in the vascular wall and in other organ systems. Additional studies are required to determine the full potential of pH_i in adjusting cell function under different physiological and pathophysiological conditions.

Initial attempts have been made to target acid-base transporters in the treatment of cardiovascular disease.

Inhibitors of NHE1 were, however, unsuccessful because of severe neurological side effects. Consequently, other targets or selective pharmacological tools are needed to inhibit acid-base transporters in a tissue-specific manner. Recent studies have shown that polymorphisms close to the gene for NBCn1 are associated with human hypertension, and NBCn1 consequently represents an attractive novel target which should be investigated for positive treatment effects in major cardiovascular conditions including hypertension and ischemic heart disease.

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