Expert Opinion

Regulation of the Na^+/H^+ exchanger in the healthy and diseased myocardium

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Background: Na^+/H^+ exchangers (NHE's) are membrane proteins that regulate ion fluxes, they extrude one intracellular proton in exchange for one extracellular sodium thereby regulating intracellular pH. Mammalian NHE's have nine isoforms, NHE1–NHE9. NHE1 is present in all mammalian cell and is the only isoform present in cardiomyocytes. NHE1 contributes to damage to the myocardium with ischemia and reperfusion and to heart hypertrophy. *Objective*: To summarize the current state of knowledge with regard to regulation of NHE1 in the myocardium. *Methods*: A review of relevant literature. *Results*: Inhibition of NHE reduces ischemia–reperfusion damage and development of hypertrophy. Extracellular-signal-regulated kinase (ERK)-dependent phosphorylation activates NHE1 in the myocardium. Ischemia and subsequent reperfusion activates the ERK-dependent pathway and may lead to aggravation of damage. *Conclusions*: Elucidation of the regulatory pathway of NHE1 in the myocardium could lead to novel approaches to reduce heart hypertrophy and ischemia–reperfusion damage.

Keywords: cation flux, Erk 1/2, hypertrophy, intracellular pH, ischemia, membrane protein, Na^+/H^+ exchanger, reperfusion

Expert Opin. Ther. Targets (2009) 13(1):55-68

1. Introduction

The mammalian Na^+/H^+ exchanger (NHE) is a ubiquitously expressed membrane protein that removes one intracellular hydrogen ion in exchange for one extracellular sodium ion. NHE plays an important role in maintaining intracellular pH (pH_i) while protecting cells from acidification, as well as regulating cell volume and sodium fluxes (reviewed in [1]). Nine isoforms of NHE exist, however, NHE isoform 1 (NHE1) is the only plasma membrane isoform expressed in the myocardium [2,3]. NHE1 is of particular importance in the myocardium as it is implicated in both myocardial damage from ischemia/reperfusion injury, and hypertrophy (reviewed in [4]). NHE1 consists of two domains, a 500-amino-acid membrane domain, which transports ions, and a cytosolic domain of approximately 300 amino acids that regulates the membrane domain and is a target of protein interactions and phosphorylation (**Figure 1**, reviewed in [5]). The exact mechanisms whereby protein kinases and phosphatases regulate NHE1 are only beginning to be understood. The purpose of this review is to summarize the current state of knowledge with regard to regulation of the NHE1 isoform of the Na^+/H^+ exchanger in the myocardium. Attention is paid to physiological and pathological roles of NHE1 in heart disease. In addition, relevant regulation of NHE1 in other tissues is discussed.

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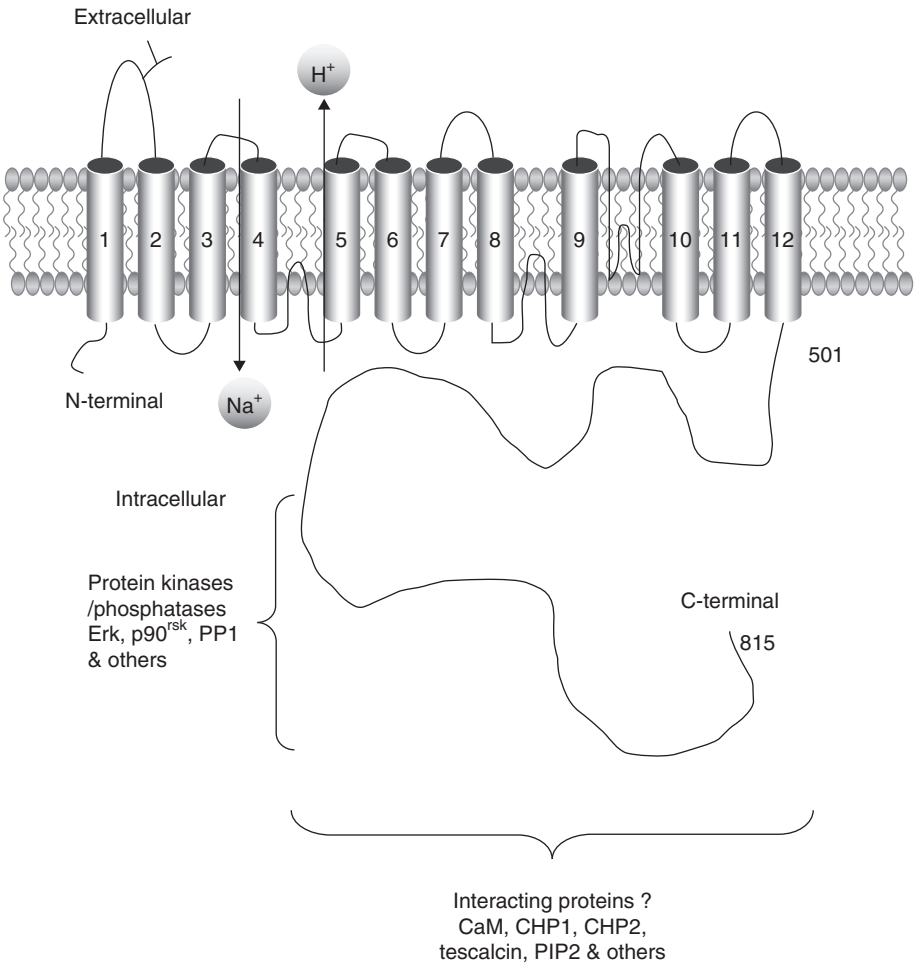

Figure 1. Schematic diagram of Na^+/H^+ exchanger 1 (NHE1) showing the membrane and cytosolic domains. Numbering and orientation of the transmembrane segments is based on [168]. The cytosolic domain is subject to modification by phosphorylation by a number of different kinases on the C-terminal 180 amino acids. A number of proteins bind to the tail region of the protein in a variety of locations. These include calmodulin (CaM), calcineurin homologous protein (CHP) isoforms, tescalcin and other proteins.
CaM: Calmodulin; CHP1: Calcineurin homologous protein type 1; ERK: Extracellular-signal-regulated kinase; ERM: Ezrin/radixin/moesin; LPA: Lysophosphatidic acid; NHE1: Na^+/H^+ exchanger isoform 1; p160ROCK: Ras homolog-associated; coiled-coil containing protein kinase; pH_i: Intracellular pH; PIP2: Phosphatidylinositol 4,5-bisphosphate; PP1: Protein phosphatase 1; rsk: Ribosomal protein S6 kinase;. SIA: Sustained intracellular acidosis;

2. Importance of NHE and pH regulation in the myocardium

NHE is important in pH_i regulation in the myocardium. With the proton generation of intermediary metabolism and the effect of negative membrane potential, protons accumulate within the cytosol and inhibit contractility. NHE1 removes these proteins and has unique activity characteristics in the myocardium compared with other tissues, with a very steep relationship between pH_i and activity [6]. At low pH_i ($pH \leq 6.5$) the exchanger is maximally active, however this pH dependence can be shifted to a more alkaline range by α1-adrenergic stimulation and by hormones including endothelin-1 [7,8]. It has been shown that MAPK-dependent phosphorylation is important in this response [9,10]. HCO_3^- based transporters contribute to recovery from intracellular acidosis, however, they generally contribute little of the total acid efflux in the myocardium. In addition they are more active at more alkaline pH's above 7.0 [11-14], so at acid pH's NHE1 is the major pH regulator [11-17].

The nucleotide sequence of human NHE1 cDNA predicts an 815-amino-acid protein. The first 500 residues are predicted to be 12 transmembrane spanning segments, and the remaining 315 residues constitute an intracellular regulatory domain [18] (**Figure 1**) although there hass recently been some doubt about the transmembrane organization [19]. NHE1 forms isoform-specific homodimers *in vivo*, although the individual dimer subunits do function independently of each other [20,21]. The structure of a bacterial NHE has been determined [22] but it is not very homologous with NHE1 and is part of a

different NHE family with different characteristics [23]. The family of mammalian NHE proteins now includes nine commonly known isoforms (NHE1 – 9) each the product of a different gene, with unique tissue distribution and physiological roles (reviewed in [5,24]). The first NHE cloned was NHE1 [25], which is ubiquitously expressed in mammalian cells and is considered to be the 'housekeeping' isoform. NHE1 was also identified by the author's laboratory as the predominant isoform in the myocardium [3], where it is concentrated along the intercalated discs and transverse tubule system [26]. Several studies used either Northern blot analysis or analysis with antibodies against various isoforms and were unable to detect significant NHE2–5 in the myocardium [27-31]. NHE2 – 4 were found predominantly in the kidney and gastrointestinal tract [27-29]. NHE5 was to befound expressed in the brain [30] while NHE6 – 9 were localized to intracellular organelle membranes, such as mitochondria, endosomes and the Golgi network [32,33]. The protein identity varies from 25 to 70%, however all isoforms share common predicted secondary structures [34]. A more distant family of two NHE proteins may be involved in mediating hypertension [35] though a splice variant of NHE1 has also been suggested to be important in hypertension [36].

3. NHE1 physiological and pathological role

NHE1 has a variety of roles in many cell types (briefly described here, see [5,23,34,37] for reviews). Knockout of NHE1 demonstrated that NHE1 plays a role in growth of cells, especially in acidic media [38]. Similarly, studies of NHE1-deficient mice showed decreased postnatal growth and the mice exhibited ataxia and epileptic-like seizures [39,40]. It has been shown that NHE1 is important in cell cycle progression [41,42]. NHE1 is also permissive in many types of cell differentiation which the author's group [43] and others [44] demonstrated. The involvement of NHE1 in cell growth, proliferation and differentiation implicates the protein as being important in normal developmental processes. Anchoring of NHE1 to the cytoskeleton via interactions with the ezrin/radixin/moesin (ERM) family of proteins also links NHE1 to a role in maintenance of cytoskeletal structure, focal adhesion and cell migration [45-47]. NHE1 is also thought to be involved in apoptosis, although its role seems to differ depending on the cells tested. In mouse β-cells, trophic factor withdrawal triggers pH_i dysregulation and apoptosis. NHE1 is activated, leading to cellular alkalinization and progression of apoptosis [48]. It has been shown that this activation is through p38-dependent phosphorylation of the NHE1 tail [48]. In contrast, another study showed different results. Re-introduction of NHE1 into NHE-null PS120 cells blunted both staurosporine- and *N*-ethylmaleimide-mediated apoptosis. In the same study it was shown that in LLCPK1 cells (kidney proximal tubule cells), NHE1 activation induced by hypertonic sucrose or acidification activated the survival kinase Akt [49] suggesting a mechanism of action of the anti-apoptotic effect.

A key feature of transformed cells is that they have an alkalinized pH_i relative to non-transformed cells, and it has been suggested that this disturbance in pH homeostasis corresponds to an increasing cancerous state [50]. NHE1 is involved in the altered pH_i of malignant cells, and NHE1-dependent alkalinization plays a pivotal role in the development of a transformed phenotype, while inhibition of NHE1 prevents it [51-53]. Furthermore, in breast cancer cells NHE1 activation has also been implicated as a key factor in breast cancer cell invasion [54-56].

4. Pathological roles of NHE1 in the myocardium

4.1 Ischemia/reperfusion

NHE1 contributes to several pathological conditions in the myocardium. The most well known is the role of NHE1 in ischemia/reperfusion damage to the myocardium [57-60]. During ischemia anaerobic glycolysis occurs resulting in the production of protons, which serve to activate NHE1. Activated NHE1 exchanges the intenal protons (H^+_i) for external sodium ions (Na^+_e) leading to a rapid accumulation of sodium in the cell [57-60]. The high sodium concentration drives the increase in Ca^{2+} via reversal of the Na^+/Ca^{2+} exchanger. The ultimate result is a buildup of Ca^{2+} in the cells triggering various pathways leading to cell death. A huge body of evidence suggests that inhibition of NHE1 during ischemia and reperfusion protects the myocardium from calcium overload (see [60,61] for reviews). In various animal models, NHE1 inhibition by drugs such as cariporide, amiloride and EMD 85131 have proven to be cardioprotective [62-64]. Activation of NHE1 regulatory pathways has also been suggested to be important in NHE1-mediated damage to the myocardium. We showed that ischemia and reperfusion activate many NHE1 regulatory kinases [10].

4.2 Hypertrophy

NHE1 is also important in cardiac hypertrophy [65]. In several models it has been shown directly that NHE1 inhibition prevents cardiac hypertrophy including *in vivo* in rats subjected to myocardial infarction [66,67], in mice with guanylylcyclase-A receptor knock out [68] and *in vitro* in isolated cardiomyocytes [69]. A number of factors that initiate hypertrophic responses have also been shown to be involved in activating NHE1 activity or are dependent upon it. For example, NHE1 is activated by MAP kinases and protein-kinase-C-dependent pathways, which are important in hypertrophic and remodeling processes [10,70]. In addition, the effect of the hypertrophic agonist aldosterone can be blocked by NHE1 inhibition [69] as can stretch-induced hypertrophy [71]. Prevention of increases in intracellular Na^+ is a good candidate for a mechanism by which NHE1 inhibition prevents hypertrophy [72,73].

4.3 Apoptosis

In the myocardium NHE1 is also important in apoptosis. Studies in animals and humans have shown that in addition to necrosis, apoptosis significantly contributes to myocyte loss following myocardial infarction [74-77], including results suggesting that apoptosis is the predominant form of cell death in infarcted human myocardium [78]. Many detrimental effects of NHE1 in hypoxia-reoxygenation are mediated or compounded by apoptosis [79-84]. Blockage of NHE1 activity via the specific inhibitor cariporide results in decreased apoptosis in isolated cardiomyocytes [85] and in fibroblasts [82]. In addition, inhibition of NHE1 activity before ischemia has been shown to reduce myocardial apopotosis in isolated rat hearts [83,84], in mouse hearts [86] and in pacing induced heart failure in rabbits [87]. Humphreys *et al.* also reported that in an ischemic rat model, the NHE1 inhibitor cariporide reduced apoptosis and this was associated with a signi-ficantly higher ratio of (antiapoptotic) B-cell lymphoma protein 2 (Bcl-2) to (proapoptotic) bcl2-associated X protein (Bax) [86,88]. Regulation of NHE1 has been implicated in NHE1-induced apoptosis in the myocardium [89,90]. Recently, we have shown that amino acids Ser^{726} and Ser^{729} are involved in critical regulation of NHE1-caused apoptosis, however this study did not examine cardiac myocytes [91].

Despite all the success with inhibition of NHE1 in experimental models, clinical trials with NHE1 inhibitors have not been very successful. Large-scale studies with several inhibitors in various types of myocardial infarctions and treatments have given varying and sometimes disappointing results. Results of recent clinical trials using NHE1 inhibition were recently reviewed [90]. Briefly, Rupprecht *et al.* [92] tested effects of cariporide in a smaller trial of patients with myocardial infarction who received coronary angioplasty. After reperfusion they found some beneficial effects on ejection fraction, wall-motion abnormalities and enzyme release. A larger scale two-stage trial [93] tested effects of the NHE inhibitor eniporide on patients with myocardial infarction. The treatment at one stage showed a dose-dependent effect of eniporide on reducing enzyme release, indicating reduced infarction. However, in a second later stage of the trial there was no beneficial effect and the overall effect was negative indicating no beneficial effect. The reason for the discrepancy between the beneficial effect in pre-clinical trials and the negative effects in this clinical trial may be due to a requirement for NHE inhibition early in ischemia, rather than in reperfusion as was the case in the clinical trial [90].

Another clinical trial, the GUARd During Ischemia Against Necrosis (GUARDIAN) trial [94], had some more positive results. Analysis of subgroups showed that cariporide was beneficial in patients undergoing coronary artery bypass graft surgery. In this trial, treatment with the inhibitor was early, which may account for its beneficial effect in this study [90]. The sodium-hydrogen EXchange inhibition to Prevent coronary Events in acute cardiac conDITIONs (EXPEDITION) trial was designed to test if pre-ischemic cariporide inhibition of NHE1 reduces myocardial injury in patients with coronary artery bypass graft surgery. Cariporide significantly reduced myocardial infarction, however it also had adverse effects and increased cerebrovascular events significantly, which resulted in the study being terminated early [90,94]. A last study [95] tested the efficacy of the NHE inhibitor zoniporideon reducing cardiovascular events in patients undergoing non-cardiac vascular surgery. There was no beneficial effect and this has been attributed to a lack of myocardial reperfusion which is required for beneficial effects of NHE inhibitors [90,95].

Based on the outcome of the animal model studies and clinical trials, it is clear that NHE1 is an important pharmacological target but that more research is necessary for success at the clinical level. One approach suggested recently is to suppress activity through intervention in regulation of the protein [90]. In this regard, it has been demonstrated that elevation of NHE1 levels in the myocardium was not harmful in itself and regulation of NHE1 was suggested to be critical for detrimental effects [96].

5. Regulation of NHE1

5.1 Hormonal regulation

NHE1 is regulated hormonally. Some of the growth factors and hormones that have been shown to activate NHE1 in many tissues include: thrombin, serum, EGF, insulin, angiotensin II, and lysophosphatidic acid [97-102], see [5,24,103] for reviews. The various extracellular agonists mediate their effects through several classes of cell-surface receptors and various signaling networks, that act to modify the C-terminal, cytosolic regulatory domain. The modifications include phosphorylation and the binding of regulatory proteins that control transport activity by altering the affinity of the transport domain for intracellular H^+ [24] (see below).

In the myocardium a variety of agonists activate NHE1 through various cell surface receptors that may be G-protein-coupled. Serum activates NHE1 but contains a myriad of agonists [9]. One serum component, Angiotensin II acts positively to stimulate NHE1 through AT1 receptor subtypes but also exerts a negative opposing action through AT2 receptor subtypes [104]. Thrombin also activates NHE1 by means of a PKC-mediated mechanism, although PKC does not directly phosphorylate the exchanger [105,106]. Endothelin activates NHE1 in cardiomyocytes [107,108] and purkinje fibres [109]. What may be the best characterized stimulation of NHE1 is via α1-adrenergic receptors. α1-adrenergic selective agonists, such as phenylephrine, increase the pH_i sensitivity of NHE1 flux [100,110-113]. The α1-adrenergic receptor-mediated stimulation of sarcolemmal NHE activity in rat ventricular myocytes requires activation of the ERK (but not the p38) pathway of the MAPK cascade [100].

5.2 Phosphorylation/dephosphosphorylation

The effects of hormonal regulation of NHE1 are mediated by protein–protein interactions and by phosphorylation of the regulatory, cytosolic domain. These have been studied principally in non-myocardial tissues, though my own laboratory has studied them in the myocardium. Various binding proteins and protein kinases interact directly and indirectly with the exchanger. Deletion analysis has revealed that the distal 180 amino acids of the 300-amino-acid carboxyl terminal tail of NHE1 are the location of phosphorylation sites of NHE1 and are responsible for about half of growth-factor-induced stimulation of NHE1 [114] (the balance of regulation of NHE1 is through protein–protein interactions, see below). **Table 1** summarizes the known protein kinases involved in NHE1 phosphorylation. Many have been shown to phosphorylate and regulate NHE1 in non-myocardial cells. ras homolog-associated, coiled-coil containing protein kinase (p160ROCK), a downstream target of ras homolog A1 (RhoA), phosphorylates NHE1 and plays a role in cytoskeletal organization [101]. Interestingly, inhibitors of p160ROCK reduce ischemia reperfusion injury in the myocardium [115]. In vascular smooth muscle cells, angiotensin II stimulates NHE1 by p38MAPK phosphorylation, and this pathway is extracellular-signal regulated kinase (ERK)-dependent [116]. We studied the kinases involved in NHE1-mediated apoptosis in pro-β cell lines. Mass spectrometry analysis revealed four p38MAPK phosphorylation sites on NHE1, Thr^{718}, Ser^{723}, Ser^{726} and Ser^{729} (human sequence) [48]. *In vivo* analysis demonstrated that these sites were important in stimulation of NHE1 in apoptosis [48]. Recent experiments have shown that Ser^{726} and Ser^{729}, are important in mediating the p38MAPK-induced apoptotic response. Also, mutation of Ser 726/729 to Ala protects cells from serum-withdrawal-induced death [91].

A variety of other kinases are involved in NHE1 regulation. These include another stress-activated kinase the Nck-interacting kinase (NIK), which binds to NHE1 [117] and Ca^{2+}/calmodulin-dependent kinase which phosphorylates NHE1 *in vitro* [106]. Other protein kinases are known to regulate NHE1 activity but not by direct phosphorylation. These include protein kinase A (PKA), C (PKC), and PKD [99,106,118,119]. Lysophosphatidic acid (LPA) is an anionic, bioactive phospholipid that mediates its effects through protein kinases. Its signaling is by associating with the G-protein-coupled receptors, LPA receptor subtypes 1 – 4 (LPA_{1-4}) [120,121]. LPA stimulates $G\alpha_{13}$ and activation of both the RhoA and Cdc42 pathways [122]. Activation of the RhoA pathway results in p160ROCK activation, leading to NHE1 stimulation, possibly by direct phosphorylation [101]. Activation of the cell division cycle 42 (Cdc42) pathway mediates ERK1/2 activation leading to NHE1 stimulation [122,123]. Very recently protein kinase B has been shown to phosphorylate Ser^{648} of NHE1 and to inhibit NHE1 activity, possibly by interfering with calmodulin binding [124]. This may be an endogenous method of physiologically inhibiting NHE1 activity [124].

ERK-dependent pathways are clearly involved in NHE1 activation in several tissue types. In vascular smooth muscle cells serum activates Erk1/2 which activates ribosomal protein S6 kinase ($p90^{rsk}$) and phosphorylates NHE1 at Ser^{703}, but in so doing, it also forms a 14-3-3 ligand binding site [125,126]. When 14-3-3 binds to the phospho-Ser^{703} it limits dephosphorylation. The hormone insulin activates NHE1 via an ERK1/2 pathway in addition to a PKC pathway [97,127]. In human erythrocytes, insulin activates phosphatidylinositol 3-kinase, which in turn activates PKCζ, and ultimately activates NHE1 [127]. Though Maly *et al.* challenge the involvement of the MAPK pathway in EGF-mediated NHE1 activation [118]. Their study only implicated PKCα in EGF-mediated NHE1 activation in mouse NIH3T3 cells. They suggest that that lack of MAPK involvement may be unique to their cell system.

It has been shown that ERK1/2-dependent pathways are critical in regulation of NHE1 in the myocardium. ERK1/2 and $p90^{rsk}$ specifically phosphorylated NHE1 and that activity of NHE1 is dependent on these pathways [9]. Also ischemia/reperfusion activated these pathways and activated NHE1 in the myocardium [10]. More recently, details of how Erk1/2 mediates NHE1 regulation in cells subjected to sustained intracellular acidosis were published. Sustained intracellular acidosis (SIA) treatment of neonatal rat ventricular myocytes results in a significant increase in NHE1 activity [128]. It acts through a Ras-, and ERK1/2-dependent pathway and activates Erk1/2 and $p90^{rsk}$ in COS-7 cells, isolated cardiomyocycte, and fibroblasts [128-130]. To localize the exact sites of Erk-dependent phosphorylation involved my colleagues and I used mass spectroscopy to identify amino acids phosphorylated by Erk1/2 [131,132]. Four domains/regions were phosphorylated comprising regions; 1, S693; 2, T718,S723/726/729; 3, S766/770/771; and 4, T779,S785. We initially characterized these regions in CHO cells devoid of their own NHE [130]. Ser and Thr were mutated to alanine residues. SIA increased NHE1 activity and phosphorylation via an ERK-dependent pathway, that was blocked with the MAPK kinase or ERK kinase (MEK) inhibitor U0126. The NHE activity and phosphorylation of mutants 1, 2 and 4 was stimulated similarly to wild type NHE1, however, mutant 3 was not. Our results showed that one or more of amino acids Ser^{766}, Ser^{770} and Ser^{771} mediate ERK-dependent activation of the Na^+/H^+ exchanger *in vivo*. More recently we have examined the role of these amino acids in the myocardium (manuscript in preparation). We made adenovirus expressing an inhibitor-resistant isoform of NHE1 and tested the effect of these mutations on activity of NHE1 in isolated cardiomyocytes. Endogenous NHE1 was inhibited with EMD87580 while the activity of the exogenous protein remained. Mutation of either Ser^{770} or Ser^{771} prevented acidosis-induced stimulation of activity and phosphorylation of NHE1 in cardiomyocytes.

We have also recently demonstrated that amino acids Ser^{726} and Ser^{729} are involved in regulation of NHE1 in

Table 1. Proteins, factors and kinases interacting with the cytosolic region of NHE1.

Protein kinase	Phosphorylation/dephosphorylation site	Other	Ref.
CaM kinase II	C-terminus	Phosphorylation shown *in vitro*	[106]
Erk1-2	Ser^{770}, Ser^{771}	Activated by sustained acidosis	[130]
$P90^{rsk}$	Ser^{703}	Serum stimulated	[166]
P38	Thr^{717}, Ser^{722}, Ser^{725}, Ser^{728} (one or more)	Activation during trophic factor withdrawal in pro-T cells	[48]
Nck interacting kinase	Binds amino acids 563 – 638 and phosphorylates distally	Activates	[117]
Protein kinase D	May act indirectly	Inhibitory	[119]
P160ROCK	C-terminus	Mediates RhoA activation	[101]
PKB/Akt	Ser^{648}	Inhibitory	[124]
Phosphatase	**Dephosphorylation site**	**Other**	**Ref.**
Protein phosphatase 1	C-terminus	Inhibitory	[133]
Protein phosphatase 2A	Unknown, co-localizes and dephosphorylates NHE1	Counteracts α1-adenosine-receptor-mediated activation	[134]
SHP-2 tyrosine phosphatase	C-terminus	Stimulatory	[135]
Protein-factor	**Binding location**	**Function/other information**	**Ref.**
Calmodulin	636 – 656	High-affinity-site stimulatory	[136]
Calmodulin	657 – 700	Low affinity site	[136]
Carbonic anhydrase IV	790 – 802	Phosphorylation-regulated, stimulatory	[151]
CHP1	518 – 537	Solution structure determined, essential cofactor	[144]
CHP2	518 – 537	Crystal structure	[167]
CHP3, tescalcin	530 – 535, second more distal binding site also reported	Promoted maturation and protein half life	[148,150]
Daxx	567 – 637	Activates	[158]
Ezrin, radixi, moesin (ERM)	552 – 560	Affects focal adhesions, stress fiber formation, cell shape apoptosis	[46,49]
Hsp70	C-terminus	May affect protein folding	[135,161]
PIP2	506 – 576 (two sites)	Mediates inhibitory effect of ATP depletion	[141]
14-3-3	C terminus, phospho-Ser^{703}	Scaffolding protein, stress	[126]
Bin1, amphiphysin I, BMX, Fas, CIDE-B, Maspin, CAS, DR4	Identified by antibody array analysis	Functional role not yet characterized	[135]

Bin I: Bridging integrator 1; BMX: Bone marrow tyrosine kinase gene in chromosome X protein; CaM: Calmodulin; CAS: Crk-associated substrate; CHP: Calcineurin homologous protein; CIDE-B: Cell-death-inducing DNA-fragmentation-factor-like effector B; DR4: Death receptor 4; ERK: Extracellular-signal-regulated kinase; Hsp: Heat-shock protein; PIP2: Phosphatidylinositol 4,5-bisphosphate; PKB: Protein kinase B; p160ROCK: Ras homolog-associated; coiled-coil containing protein kinase; RhoA: Ras homolog A1; rsk: Ribosomal protein S6 kinase; SHP: SH2-domain containing protein.

apoptosis. We identified these phosphorylation sites on NHE1 that regulate its alkalinizing activity in response to a cell-death stimulus. Performing targeted mutagenesis, we observed that substitution of Ser^{726} and Ser^{729} for alanines produced a mutant form of NHE1 that did not alkalinize in response to an apoptotic stimulus, and expression of which protected CHO cells from serum-withdrawal-induced death [91].

Protein phosphatase 'dephosphorylation' of NHE1 is only beginning to be studied. My colleagues and I [133] showed that treatment of primary cultures of cardiomyocytes with the phosphatase inhibitor okadaic acid increased the rate of recovery from an acid load, suggesting that the okadaic-acid-sensitive protein phosphatase 1 (PP1) may be involved in NHE1 regulation *in vivo*. Furthermore, we demonstrated that PP1 dephosphorylates the regulatory cytoplasmic tail of NHE1 and associates with NHE1 both *in vitro* and *in vivo* [133]. Other protein phosphatases that have been suggested to be significant in NHE1 regulation are protein phosphatase 2A [134] and SH2 domain-containing protein tyrosine phosphatase (**Table 1**) [135].

5.3 Regulatory proteins

A number of regulatory proteins and cofactors bind to the NHE1 tail and probably mediate the 50% of hormonal regulation that is not due to phosphorylation (**Table 1**) [114]. They have not been well studied in the myocardium – mostly they have been examined in other tissues, but will be reviewed here both to get a greater understanding of how NHE1 and to understand which areas that may be the subject of future investigation in the myocardium. Calmodulin (CaM) is one protein regulator of NHE1. It is a second messenger calcium-binding protein which plays an important role in modulating NHE1 activity in response to intracellular Ca^{2+}. After binding Ca^{2+}, CaM binds to two CaM binding domains in the cytoplasmic tail of NHE1, a high- and a low-affinity site located between amino acids 636 – 700 [136]. Residues Leu^{639}, Lys^{651}, and Tyr^{652} are particularly important in the interaction [137].Binding to the high-affinity site, blocks the auto-inhibitory interaction and thereby activates NHE1 [138]. Additionally, we have shown that seven conserved acidic amino acids, ^{753}EEDEDDD^{759}, in the distal region of the C-terminal tail play a role in modulating CaM binding [139].

NHE1, although not directly consuming ATP, is affected by cellular ATP depletion [140]. Phosphatidylinositol 4,5-bisphosphate (PIP_2) reduction is concurrent with ATP depletion and depletion of PIP_2 results in the NHE1 inhibition [141]. This ATP-dependence is due to an association with two PIP_2-binding motifs in the cytoplasmic tail of NHE1 between amino acids 513 and 520 and 556 and 564 and mutation of these sites reduces NHE1 activity. PIP_2 was shown to bind the C-terminal tail of NHE1 *in vitro* in these regions [141].

Calcineurin homologous protein 1 (CHP1) exists in two well-known isoforms, 1 and 2, which share 61% amino acid identity [142,143]. These proteins possess four EF-hand motifs, although only EF3 and EF4 actually coordinate Ca^{2+}. CHP1 is an essential cofactor for NHE1 activity and binds to NHE1 at amino acids 518 – 537, and CHP1 mutants that impede binding result in a dramatic loss of Na^+/H^+ exchange activity [144]. CHP1 is present in the myocardium [145]. CHP1 has been shown to interact with and inhibit the apoptotic kinase death-associated protein kinase-related 2 (DRAK2) [146,147]. CHP2 is expressed at high levels in tumor cells and the association of CHP2 with NHE1 protects cells from serum deprivation-induced death by increasing pH_i. It is proposed that the CHP2–NHE1 association maintains the malignant state of transformed cells. CHP2 mRNA has been detected in the myocardium but it has not been studied there [143].

A protein related to CHP1 and CHP2 is tescalcin (sometimes called CHP3). It is a Ca^{2+} binding protein that is homologous to CHP1. Tescalcin is strongly expressed in the myocardium where it binds to the cytoplasmic tail of NHE1 in a Ca^{2+}-dependent manner [148,149]. It was shown that its binding affects the conformation of the cytoplasmic tail [148] and it is reported to promote maturation and cell surface stability of NHE1 in CHO cells [150]. Its role in the myocardium has not been investigated.

Carbonic anhydrase II (CAII) is an enzyme that catalyzes the production of HCO_3^- and H^+ from the hydration of CO_2. We showed that CAII associates with NHE1 *in vivo* via interaction at residues 790 – 802 of the C-terminal tail with Ser^{796} and Asp^{797} forming part of the binding site [151]. Association of these two proteins increases NHE1 activity, and is dependent on the phosphorylation state of NHE1 [152]. It appears as though a region upstream of the CAII binding site, when dephosphorylated, interferes with CAII binding [151]. The association of CAII with NHE1 may result in more efficient removal of protons that are produced by CAII activity. Though it was previously suggested that CAII was not present in the myocardium, it was recently shown to be present in isolated cardiomyocytes [153] and CAII inhibitors reduced NHE1 activity in the myocardium [151,153].

NHE1 is linked to the cytoskeleton through ezrin, radixin, and moesin (ERM). These are a family of proteins which form links between actin filaments of the cytoskeleton and integral proteins of the plasma membrane [154]. NHE1 has two ERM binding motifs in its cytoplasmic tail between amino acids 552 and 560, and this interaction directs proper localization of NHE1 and maintains cell shape [155] and is important in cellular events such as cell migration, formation of signaling complexes [45,46,49]. In fibroblasts and human renal proximal tubule cells, it was shown that apoptotic stress activates NHE1, and NHE–ERM interaction is required for cell survival signaling. The NHE1-ERM complex associated with the cytoskeleton and activated the pro-survival kinase Akt. This complex was necessary for Akt-enhanced cell survival. The NHE1 and ERM complex activate the pro-survival kinase, Akt, and stall apoptosis [49]. Since NHE1 seems to be proapoptotic in cardiomyocytes [85,88] this seems to be a pathway that does not function in the myocardium and

the absence of ERM and ERM-associated proteins in the myocardium [156,157] supports this suggestion.

Daxx is a death domain associated protein implicated in ischemic cell death [158]. Daxx was found to bind to the ERM-interacting domain of NHE1 in competition with ezrin. In PS120 cells (fibroblasts) the Daxx–NHE1 interaction was shown to mediate ischemia-induced cell death [158]. Daxx is present in neonatal rat ventricular cardiomyocytes [159] and expression of a dominant-negative Daxx in the myocardium reduces ischemia/reperfusion damage [160].

14-3-3 adaptor protein (described above) binds to NHE1 only when it is phosphorylated on Ser^{703}. This activates NHE1 by protecting Ser^{703} from dephosphorylation [126].

My colleagues and I [161] demonstrated that heat-shock protein (Hsp)70 also binds to the C-terminus of NHE1 and may participate in folding of the protein. This was subsequently verified by another laboratory [135]. That study also showed that a number of other proteins (amphiphysin I, bridging integrator 1 (Bin I), Bone marrow tyrosine kinase gene in chromosome X protein (BMX), Fas, cell-death-inducing DNA-fragmentation-factor-like effector B (CIDE-B), Maspin, Crk-associated substrate (CAS), death receptor 4 (DR4)) were potential binding partners of the NHE1 C-terminus, but these were not characterized in detail. Some [162-164] (Amphiphysin I, BMX) but not all of these proteins are reported to be present in the mammalian myocardium.

6. Expert opinion

NHE1 is a pH regulatory protein that is important in many aspects of cardiovascular physiology and pathology. It regulates pH_i in the myocardium and is involved in several pathologies including hypertension, cardiac hypertrophy, apoptosis and ischemia and reperfusion damage to the heart. NHE1 is clearly regulated by ERK-dependent pathways and these may be involved in mediating some of the detrimental effects of NHE1. It is clear that there is currently a reasonable understanding of the fundamentals of how the protein works in some tissues. Regulation of the NHE1 protein has been studied in several tissues. However, given the importance of the NHE1 protein in the myocardium a greater emphasis on the regulation of the NHE1 protein is of keen interest. NHE1 has important roles in ischemic damage to the heart, heart hypertrophy and apoptosis. In addition it has roles to play in uncontrolled cell growth. A number of kinases and phosphorylation sites of the protein have been identified and proteins have been shown to be involved in regulation of NHE1. Nevertheless, the role of these kinases and proteins in specific tissues and in the disease state, has not been well studied. This is of particular importance since studies have suggested that the absolute level of NHE1 may not be critical to its role in heart disease, but rather, the regulated activity of the protein may be more influential. These goals can be achieved by a more directed approach to the study of the protein. Working, for example, with the study of the regulation of the protein in isolated cardiomyocytes, a difficult cell type to study.

Another area of great benefit may be the development of improved inhibitors of NHE1. As noted above, inhibition of NHE1 has been shown to be beneficial in the disease state in a multitude of animal studies. However, in treatments with humans the effectiveness is not that impressive and significant side effects have been noted. It is not known whether the side effects observed were due to effects on the NHE1 protein, or due to effects on other proteins. Certainly it would be desirable to investigate the cause(s) of the side effects, as there were benefits of NHE1 use [60,165] that could, provide important clinical treatments. The goal of improved NHE1 inhibitors would probably be achieved through industrial efforts, modifying existing inhibitors with thorough follow up of new compounds. In addition, elucidation of the three dimensional structure of the protein may allow for improved drug design based on the elucidated structure.

Acknowledgements

The author receives support from the Canadian Institute of Health Research and the Alberta Heritage Foundation for Medical Research.

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