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Review

The Na–H exchanger revisited: an update on Na–H exchange regulation and the role of the exchanger in hypertension and cardiac function in health and disease

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1. Introduction

The Spotlight Issue of *Cardiovascular Research*, published in early 1995, was dedicated to various aspects of the Na–H exchanger (NHE) as they pertain to the cardiovascular system. It is appropriate to state that since the publication of that issue major strides have occurred which serve to enhance our understanding and appreciation of the importance of this major pH regulatory process in the cardiovascular system, both with respect to normal homeostasis as well as pathology. This brief revue has been written in order to provide an 'update' on developments in this active field since the publication of the *Cardiovascular Research* Spotlight Issue, again with a focus on the cardiovascular system in health and disease. The review is not intended as an exhaustive treatment of this topic, but rather it concentrates on recent developments and, accordingly, the authors have relied primarily on publications which have appeared in 1994 and later, except when an earlier article was deemed appropriate to reinforce a particular concept. For readers interested in comprehensive discussion of the NHE, particularly with respect to cardiovascular function, a number of recent reviews [1–4] and a monograph [5] can be recommended.

2. NHE isoforms

At the time the reviews of the Spotlight Issue on the NHE were prepared, four exchanger gene isoforms were known to exist in mammals. This list has now been expanded by a fifth NHE gene and possibly even a sixth. NHE-1 has been recognized for some time as the ubiquitous 'housekeeping' isoform. It participates in the regulation of cytoplasmic pH and volume of the cells, which includes, of course, the cardiac myocyte (but is not the sole mechanism responsible for these functions). The function of NHE-2, which is found in renal and intestinal epithelia, is still is not well understood although it had been suggested to participate in epithelial volume regulation. NHE-3 occurs in the apical membrane of epithelia and is generally held responsible for the apical entry of sodium ions in trans-epithelial Na transport. NHE-4 is found mainly in the stomach and kidney, with an ill-understood role. NHE-5 is known only in the form of a partial genomic clone [6]. Its message is found mainly in brain and testis, but again nothing is known yet about its physiological role. Finally, hybridization studies intended to provide chromosomal assignments in man have demonstrated the presence of two genes on separate chromosomes that are recognized by NHE-3-specific probes. One gene is probably NHE-3, while the other one could be a either a pseudogene related to NHE-3 or yet another isoform [7,8]. Among these NHE isoforms, NHE-1 is the sole isoform detectable in the cardiac myocyte [9] and its function and regulation has the greatest relevance for cardiovascular physiology and pathology. In the context of this update on NHE and cardiovascular function, we will therefore focus almost exclusively on NHE-1.

3. Regulation of NHE function

The human NHE-1 protein is 815 residues in length and consists of two major domains. The first approximately

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500 amino acids make up the membrane-intrinsic domain while the remaining amino acids make up the cytoplasmic domain. The membrane-intrinsic domain, which contains on the order of 12 membrane-spanning segments, mediates the NHE function, and the hydrophilic cytoplasmic domain contains the elements through which transport activity is modulated [10]. The different isoforms are highly homologous in the membrane-intrinsic domain but differ considerably in the cytoplasmic domain [11], which is consistent with the observations that the different isoforms are differentially regulated by different stimuli [12,13].

The activity of NHE-1 can be modulated by a number of growth factors, hormones and neurotransmitters (via receptor tyrosine kinases and seven-transmembrane receptors coupled to G proteins), as well as a by hypertonic shrinking and mechanical stimuli [5]. Most of these signals stimulate NHE by shifting the pH_i-activity curve towards the alkaline range, thus stimulating the enzymatic activity of the transporter at constant pH_i and moving it closer to its maximal rate. It had been demonstrated early on by Pouysségur's group that at least some of the stimulatory signals lead to phosphorylation of the exchanger protein (reviewed in [14]). While the exchanger is constitutively phosphorylated in the unstimulated cell, the extent of phosphorylation on serine residues is increased with about the same time course as the increased transport activity. The phosphorylation sites were recently mapped to the C-terminal domain of NHE-1 by use of C-terminal deletion constructs [15]. When the C-terminus beyond position 698 was deleted, the serum-stimulated phosphorylation was no longer observed; and when the C-terminus beyond position 635 was eliminated, the constitutive phosphorylation was virtually eliminated as well.

However, phosphorylation is not the sole mechanism by which NHE-1 is regulated. This has become clear from mutational and deletional analyses along the same line [14–17]. Although deletion of the C-terminus after residue 698 removed all sites that are phosphorylated in response to activation by a combination of thrombin/insulin, more than half of the transport activation remained. Conversely, deleting the stretch between positions 567 and 635 eliminated transport stimulation but preserved mitogen-stimulated phosphorylation [17]. These data demonstrate that important additional signalling elements, which are independent of NHE-1 phosphorylation, participate in the activation of transport function. This conclusion is supported by the recent observation that in salivary acinar cells carbachol activates NHE without increasing the extent of NHE-1 phosphorylation beyond the unstimulated level [18].

The best characterized and possibly most important phosphorylation-independent mechanism involves an interaction between calmodulin and the cytoplasmic domain of the exchanger protein. This interaction of calmodulin with NHE had been sketched out in the Spotlight Issue [10] but was not yet referenced. The experimental evidence in favour of this scheme comes from the studies of the Pouysségur group [17,19] which were published at about the same time as the Spotlight Issue. These experiments demonstrated that 'region A', a stretch between residues 636 and 656, can bind Ca-calmodulin with a high affinity. The authors proposed that in the unstimulated state this region A functions as a self-inhibitory domain. However, when the Ca concentration is elevated and induces calmodulin to bind to region A, the autoinhibitory effect is abolished. At the present time, this mechanism of Ca-calmodulin activation appears to be able to account for most, if not all, of the Ca-dependent activation of NHE, although it is generally understood that increased Ca levels could also modulate the activity of kinases which might be members of signalling cascades that activate NHE.

There is evidence for interactions between NHE-1 and additional proteins besides calmodulin. One line of evidence involves the long-standing observation that cytoplasmic ATP depletion leads to reduced transport activity in many cells including cardiac cells [20,21]. It was recently demonstrated that even though ATP depletion causes inhibition of transport, the extent of NHE phosphorylation is unaffected [22]. Furthermore, deletion mutants lacking the C-terminus after position 635 or 566 no longer exhibited the strong transport inhibition upon ATP depletion. Thus a region near the C-terminus of the cytoplasmic domain confers the sensitivity to ATP, possibly through an associated protein whose binding is modulated by the ATP level.

An NHE-1 accessory protein of 24 kDa in size has recently been isolated by co-immunoprecipitation using antibodies against NHE-1 [23]. This protein could be immunoprecipitated with NHE-1 independent of whether Ca was present or absent. Also, the protein could be immunoprecipitated using a NHE-1 deletion mutant that lacked the calmodulin-binding region A (residues 567–635). Thus this protein is not calmodulin. Other candidate proteins were excluded with the help of protein-specific antibodies, such as against a small heat shock protein. On the other hand, one of the larger heat shock proteins, hsp70, has been found to be associated with NHE in an independent immunoprecipitation study [24]. At the present time, the potential role of any of these proteins in modulating exchanger activity, if there is any, is not known.

3.1. Intracellular signalling intermediates involved in NHE-1 regulation

NHE is regulated by a network of intracellular signalling pathways which differ in their details among the different cell types and for the different NHE isoforms. Much of the recent work has focused on determining which of the known components of the different signalling pathways modulate NHE. These studies highlight the increasingly sophisticated molecular-biological tools that have become available in the past few years. These were made possible because of the availability of artificially introduced mutations of clones which either led to consti-

tutively activated signalling components (such as the GT-Pases) or which had a negative dominant effect (such as MAP kinase). As a caveat, these studies made extensive use of fibroblasts derived either from chinese hamster lung (CCL39) or chinese hamster ovary (CHO) since NHE-deficient cell lines are available (PS120 and AP-1, respectively) for transfection studies with NHE-1 mutants. As will be evident below, observations made with these cells do not necessarily apply to all other cell types and are therefore best considered as guides for studies of the regulation of NHE-1 in cardiac cells.

Among the receptor-coupled G proteins, $G\alpha_q$, $G\alpha_{12}$ and $G\alpha_{13}$ have recently been found to be involved in the modulation the activity of NHE-1 [25–28]. Of these three, $G\alpha q$ is coupled to phospholipase C (PLC) [26], whose activation produces diacylglycerol, the activator of protein kinase C (PKC) with its well-known effect on NHE activation [5], and inositol trisphosphate which in turns triggers Ca release and activates calcium-dependent pathways (including the above-mentioned Ca-calmodulin). However, activation of protein kinase C by G_q is not the sole pathway to activate PKC. For example, platelet-derived growth factor (PDGF) was found to activate NHE in PDGF receptor-transfected CHO cells by directly activating a different phospholipase C isoform. In addition, the PDGF receptor also activated directly a phosphatidylinositol kinase which ultimately led to activation of NHE [29].

$G\alpha_{12}$ and $G\alpha_{13}$ were also found to influence NHE-1 activity. Interestingly, the effect of $G\alpha_{12}$ depends on the cell type. In COS-1 cells it stimulates transport [25], whereas in HEK293 and CCL39 fibroblasts it inhibits NHE-1 but activates NHE-2 and NHE-3 [30]. On the other hand, $G\alpha_{13}$ stimulates all three isoforms [30]. In CCL39 cells, $G\alpha_{13}$ stimulates NHE through at least two parallel pathways involving different small GTPases of the Rho subfamily: Cdc42 and Rho [28]. A third member, Rac, appears to feed independently of $G\alpha_{13}$ into the same cascade as Cdc42 [28]. There is also a curious reciprocity between Rho and NHE-1: When NHE-1 is inhibited by the NHE inhibitor, HOE694, or in the absence of exchanger in an NHE-1-deficient cell line, $G\alpha_{13}$ cannot induce the Rho-dependent formation of stress fibres [31]. These pathways run at least in part separately from the cascade utilized by yet another GTPase, Ras. Triggered by a G protein, Ras mediates the serine/threonine kinase Raf, which in turn causes a kinase to phosphorylate p42/p44 MAP kinase, but not p38 MAPK [15]. In CCL39 fibroblasts, the p42/p44 MAPK pathway appears to be the predominant pathway through which growth factors stimulate the exchanger. However, despite this broadened knowledge about different signalling cascades converging on NHE-1, it is not yet known which kinase actually phosphorylates NHE-1 at its serine residues. Although NHE-1 possesses consensus sequences for MAP kinase, the experiments of Bianchini et al., 1997 [15] indicate that NHE is not phosphorylated by this kinase.

At least one more important intracellular signalling chain awaits identification, namely that mediating the response to a hypertonic challenge. None of the signalling components discussed above can account for the activation of NHE by shrinkage [15]. NHE is activated by hypertonicity in a Ca-independent manner [32,33]. Also, deletion of the phosphorylation sites on the cytoplasmic domain after position 698 had no influence on hypertonic stimulation of transport [34]. Experiments with more drastic C-terminal deletions suggest that hypertonic stress might relieve the auto-inhibitory effect of domain A, and that an additional volume-responding site exists in a region closer to the N-terminus [34]. A possible candidate mechanism for shrinkage-mediated exchange activation involves myosin light chain kinase (MLCK) [32]. However, this model needs to explain how Ca-calmodulin, which is needed to activate MLCK, can do that with an essentially undetectable change in intracellular Ca levels.

3.2. Transcriptional regulation of NHE-1

Some of the regulators of NHE-1 activity influence NHE not only by modulating the activity of existing transporters but also by raising the levels of mRNA through transcriptional regulatory mechanisms as exemplified by the recently described effect of $G\alpha_q$ and $G\alpha_{13}$ [26]. With the cloning of the promoter region of the NHE-1 gene from several species it has become possible to start investigating the regulatory elements participating in transcriptional regulation of NHE-1. The first demonstration of NHE-1 promoter activity was provided by Kolyada et al., 1994 [35]. By footprinting analysis they also demonstrated the existence of four protected sites (binding regions A through D) that could bind proteins from liver nuclear extracts. Subsequent experiments showed that one of these sites (binding region D) was selective for the family of C/EBP proteins, a group of CCAAT-enhancer-binding proteins which has a high abundance in liver cells but which was also active in the smooth muscle cell line tested [36]. Binding region A contained the TATA box, and binding regions B and C were characterized by Fliegel and coworkers as containing a binding site for AP-2 or an AP-2-like transcription factor [37]. The AP-2 site is responsible for much of the NHE-1 gene activation during the differentiation of embryonal carcinoma (P19) and myoblast (L6) cells [38,39]. It also contributes to the transcriptional activity in cardiomyocytes, although about 75% of the promoter activity in these cells is due to elements distal to the AP-2 site [40]. Finally, Fliegel's group characterized yet another element, consisting of 15–20 dT residues, which appears to play an important role in regulation of promoter activity in L6 cells and 3T3 cells [41].

4. Role of NHE in hypertension

In the past few years there has been growing evidence suggesting a relationship between primary hypertension

and the NHE activity in platelets, leucocytes and erythrocytes as well as vascular smooth muscle cells (VSMC, reviewed in [42]). There are many possible explanations for this phenomenon. These include systemic factors (circulating hormone levels, metabolic acidosis, salt intake), NHE-intrinsic factors (allelic differences in NHE protein expression or activity, and intracellular factors, i.e. alterations in intracellular signalling) [42]. Recent evidence points in the direction of a change in intracellular signalling.

To begin with, there is the observation that lymphocytes from hypertensive patients retain their elevated NHE activity even after they have been immortalized to a permanent cell line [43,44]. At least in this cell model, this argues against the notion that external factors such as altered hormone levels, which are either responsible for or the result of hypertension, stimulated the exchanger. Other experiments in which the entire open reading frame of NHE cDNA from normotensive and hypertensive patients was determined, eliminated the possibility that changes in the protein's structure had influenced its function. Furthermore, the mRNA levels for NHE-1 are the same for normotensives and hypertensives in immortalized human lymphocytes [43] and rat VSMC (normotensive control WKY rats vs. hypertensive SHR rats) [45,46] and so are the NHE-1 protein levels in rat VSMC [47]. Interestingly, differences were only found in VSMC derived from aorta but not from pulmonary arteries [48].

The differences in NHE activity might be due to differences in the activation state of NHE-1 protein. Hoffman and colleagues [49] found that phorbol ester stimulated NHE in VSMC from WKY rats but not from SHR rats and that the kinetic properties of the phorbol-stimulated WKY exchanger were the same as those of the SHR exchanger, as if the latter were in a constitutively stimulated state. In an apparent contradiction, a separate study reports that phorbol ester stimulated NHE in VSMC from WKY rats more strongly than in cells from SHR rats [50]. The same group did find that increasing the intracellular Ca level using an ionophore stimulated NHE-1 activity only in VSMC from WKY but not SHR [51]. The resting phosphorylation level of NHE protein in cells from WKY rats was found to be only one-half of that in SHR rats [50,51]. The physiological significance of this observation is not clear since it appears that in VSMC at least the acute regulation by calcium is phosphorylation-independent and probably utilizes the regulatory effect of calmodulin [51].

In a manner analogous to what was found in VSMC, the exchanger in immortalized lymphocytes from hypertensive subjects exhibits a higher level of phosphorylation than that of normotensive controls [52]. However — and this symbolizes the present lack of a consistent picture in this field and emphasizes that not all contributing factors have been found yet — normotensive subjects with a family history of hypertension also exhibited an elevated state of phosphorylation [52]. In general, it is not even clear what the physiological connection is between hypertension and excessive NHE-1 activity in blood cells or VSMC. Since the ultimate determinant of blood pressure is the retention of salt and water by the kidney, one could argue that the altered systemic NHE-1 activity reflects that of the renal exchanger. Consistent with this notion is the finding that a recently developed transgenic mouse in which NHE is constitutively overexpressed, develops salt-sensitive (but not essential) hypertension [53]. However, the NHE responsible for NaCl reabsorption in the kidney is the NHE-3 isoform [54]. One would therefore expect that if a Na–H exchanger contributes to hypertension, it would be NHE-3. Indeed, expanding on older findings of increased renal brush border NHE, a recent report describes increased NHE-3 protein levels but equal NHE-1 protein levels in freshly isolated renal tubule cells from SHR compared to WKY rats [55]. One might speculatively reconcile this conflict by postulating that the factor(s) that upregulate NHE-3 expression in the renal brush border also stimulate NHE-1 transport activity in other cells. However, if such a factor exists, it is not maintained in the renal tubular cells after prolonged culture, thus leading NHE-3 levels to return to control levels [55].

An important question to consider is whether increased NHE activity in hypertension is associated with the development of the disease. An interesting corollary has recently been reported where angiotensin converting enzyme (ACE) inhibition with quinapril reversed the increased NHE activity in lymphocytes of patients with essential hypertension although NHE-1 expression (which was identical in normotensive and hypertensive patients) was unaffected [56]. However, whether this property of ACE inhibition contributes to therapeutic effects of this class of drugs remains to be determined.

5. Role of NHE in regulation of cardiac function

As previously reviewed [57], pH can exert profound effects on cardiac function and mechanisms must exist to remove protons and maintain intracellular pH at physiological levels. In this regard NHE represents a major route of proton extrusion in the maintenance of a normal intracellular pH, although other regulatory processes such as bicarbonate-dependent transporters are also of importance. The fact that the cardiac cell possesses several pH-regulatory systems is of immense importance with respect to the use of NHE inhibitors as therapeutic tools, because inhibition of the antiporter does not totally abolish the movement of acid equivalents, particularly under conditions of proton loading such as during ischemia.

There is now emerging evidence that NHE activation does not occur only as a result of proton generation but also as a consequence of receptor-linked mechanisms possibly via phosphorylation reactions. Thus, positive inotropic agents such as endothelin-1 (reviewed in [58]),

α_1-adrenergic agonists [59] (reviewed in [60]) and angiotensin II [61] can stimulate cardiac NHE, which could explain their positive inotropic effect as a consequence of intracellular alkalinization and the resultant myofibrillar sensitization to calcium. However, recent evidence using the feline myocardium suggests that NHE activation does not mediate the positive inotropic effect of angiotensin II as the effect was not blocked by ethylisopropylamiloride [62].

Recently, it has been shown that thrombin stimulates NHE in rat ventricular myocytes through receptor-linked mechanisms possibly involving protein kinase C-dependent phosphorylation [63]. As thrombin exerts numerous effects which could be important in both the normal and diseased myocardium, these findings suggest a potential role of NHE in mediating some of these actions.

6. Cardioprotective effect of NHE inhibition

An impressive observation with respect to all studies using NHE inhibitors is the consistent finding that they exhibit cardioprotective properties irrespective of experimental design, type of animal species, route of drug administration or parameter under study (reviewed in [1–3]). Studies published within the past few years have strongly reinforced the concept of NHE involvement in myocardial ischemic and reperfusion injury. Earlier studies have relied to a large degree on the use of amiloride or amiloride analogues to assess the role of the antiporter in tissue injury. Their conclusions, which are based on the assumption that the amiloride effect is mediated by its specificity for NHE, are reinforced by the more recent studies in which a different class of highly potent NHE inhibitors with dissimilar structure (HOE 694 and HOE 642) was used.

6.1. Studies with amiloride analogues

Work with amiloride analogues such as ethylisopropylamiloride has demonstrated excellent protection when administered prior to ischemia and reperfusion in isolated rat hearts or coronary artery ligation in vivo as manifested by reduction in arrhythmias, reduced cell injury and preservation of energy metabolites [64]. Moreover, ethylisopropylamiloride produced nearly an 80% reduction in infarct size in rabbits subjected to myocardial ischemia and reperfusion [65]. Interestingly, ethylisopropylamiloride failed to exert salutary effects when administered during reperfusion, a finding supporting the concept held by some investigators that the beneficial effects of NHE inhibition requires pretreatment. From a mechanistic point of view, it is suggestive of an important role of NHE during ischemia in mediating tissue injury. Indeed, this concept has been reinforced by the recent study of Koike et al. [66] who demonstrated using ^{31}P nuclear magnetic resonance spectroscopy that administration of the drug during ischemia and reperfusion in isolated ischemic and reperfused rabbit hearts resulted in greater acidification during ischemia and slower recovery from acidosis after reperfusion; these were associated with diminished contracture development and enhanced systolic recovery. Although the accentuation of ischemia-induced acidosis by dimethylamiloride is strongly suggestive for a role of NHE in pH_i regulation during ischemia per se, other investigators have failed to demonstrate this effect of ethylisobutylamiloride on pH_i despite the ability of this drug to attenuate sodium loading and improve ventricular recovery [67].

Another potentially important site of action of NHE inhibitors which could be particularly important when utilizing in vivo approaches is inhibition of neutrophil-induced cardiac injury. In one related study it was found that methylisobutylamiloride reversed the deleterious effects of added neutrophils to isolated ischemic and reperfused hearts [68].

6.2. Studies with HOE 694 and HOE 642 (cariporide)

As noted previously, the most recent studies have utilized the HOE compounds to delineate NHE involvement. For example, in the first characterization of HOE 694, this compound exerted protective effects in terms of diminishing the incidence of arrhythmias, preserving energy metabolites and reduction in cell damage in isolated ischemic and reperfused rat hearts and markedly reduced arrhythmias in rats subjected to coronary artery ligation including a complete suppression of fibrillation [69]. In studies using isolated blood-perfused rabbit hearts HOE 694 exerted protective effects when administered prior to ischemia in terms of reduction in contracture, improved systolic function and preservation of ATP levels [70] with only moderate protection when administered only upon reflow. HOE 694 has been shown to significantly reduce infarct size and preserve segmental shortening in a porcine model of myocardial ischemia and reperfusion [71]. An intravenous bolus of HOE 694 to either dogs or rats subjected to coronary artery ligation and reperfusion revealed a significant reduction in the incidence of ventricular tachycardia and fibrillation as well as mortality [72]. Moreover HOE 694, administered as a bolus before ischemia, completely prevented ventricular fibrillation, improved myocardial function and preserved ultrastructural integrity after reperfusion in a porcine model [73]. The antiarrhythmic properties of HOE 694, as well as ethylisobutylamiloride, have also been well-demonstrated in an isolated rat heart model of ischemia and reperfusion resulting in ventricular fibrillation [74]. Interestingly, in that study both agents were equally effective as antiarrhythmic agents when administered only at reperfusion. These investigators also proposed that buffer composition may serve to explain some of the controversy concerning the locus of action of NHE inhibitors, i.e. during ischemia or reperfusion. They reported that HOE 694 as well as dimethylamiloride exerted protective effects irrespective of

time of addition when hearts were perfused with bicarbonate-free medium whereas addition before ischemia was a prerequisite for protection with bicarbonate-containing medium [75]. When applied to the in vivo situation it appears that pretreatment is important for the demonstration of cardioprotection with NHE inhibitors. For example, HOE 642 was recently shown to significantly decrease infarct size in a rabbit infarct model when administered prior to ischemia but not when given prior to reperfusion [76].

The potential cellular basis for the antiarrhythmic properties of NHE inhibitors has recently been studied using electrophysiological techniques in a variety of preparations including isolated rat and rabbit hearts as well as rabbit ventricular myocytes. In that report [77], the antiarrhythmic properties of methylisobutylamiloride were associated with maintenance of various sodium and calcium entry mechanisms including prolongation of action potential duration and prevention of the appearance of transient inward currents. Thus, it is possible that despite the fact that NHE is an electroneutral process, it may indirectly affect ionic currents regulating cardiac function, particularly under pathological conditions.

6.3. Interaction between NHE inhibitors and other cardioprotective agents

Studies have also been carried out to assess potential additive protective effects of NHE inhibitors with other cardioprotective strategies. For example Yamada et al. [78] showed that amiloride enhanced the protection afforded by reduction of extracellular sodium and calcium concentrations in isolated working rat hearts subjected to 30 minutes of cardioplegic arrest. Moreover, in isolated working rat hearts subjected to ischemia administration of amiloride in combination with the hydroxyl radical scavenger desferrioxamine produced superior cardioprotective effects compared to each drug alone [79]. These results are therefore suggestive of a specific and distinct target for the beneficial effects of NHE inhibition, rendering this approach attractive for potential superior cardioprotective strategies using drug combination protocols. Moreover, additive protective effects of HOE 642 are observed when the drug is administered in combination with either of the volatile anaesthetics sevoflurane or isoflurane in isolated ischemic and reperfused rat hearts [80]. Although indicative of distinct mechanisms of action, these findings also suggest that the combination of HOE 642 and these volatiles produces superior cardioprotection which may be of importance under clinical conditions where effective cardioprotection is desired during surgical procedures.

6.4. NHE inhibition and cardiac protection during prolonged hypothermic storage

Inhibition of NHE may represent an attractive and effective approach towards long-term cardiac preservation which may be important in improving techniques for heart storage prior to transplantation. In this regard, rabbit hearts subjected to 12 hours of hypothermic (4°C) arrest demonstrated significantly enhanced functional recovery accompanied by diminished contracture development when treated with HOE 694 [81]. Interestingly, in this model of cardioplegic arrest the protective effects of HOE 694 were also evident when the drug was administered immediately before reperfusion, thus preischemic administration was not a prerequisite to demonstrate protection. Although this may be in contrast to studies utilizing acute ischemic conditions (see below) the results likely reflect the fact that in this model, hearts were protected by the cardioplegic hypothermic conditions during the 12 hour period thus precluding any potential beneficial effects during this period. Similar results were obtained by Kupriyanov et al. [82] who showed that amiloride attenuated functional and metabolic impairment produced in hearts subjected to 15 hours of hypothermic arrest (10°C). Surprisingly, one group of investigators demonstrated a deleterious effect of amiloride on functional recovery after 12 hours of hypothermic storage despite the drug's ability to attenuate sodium loading during ischemia, although the basis for this apparent contradictory finding is not known [83].

7. Mechanism of NHE involvement in cardiac injury

The basis for NHE involvement in myocardial ischemic and reperfusion injury most likely reflects the inability of the ischemic cardiac cell to extrude sodium due to inhibition of the Na–K ATPase, at the same time when NHE is activated to extrude protons under acidic conditions. The combination of these factors produces elevated intracellular sodium levels which in turn increases intracellular calcium levels via Na–Ca exchange (reviewed in [1–3,84]). This concept has been reinforced in studies using reoxygenated cardiomyocytes in which HOE 694 reduced, whereas ouabain increased cell injury as manifested by hypercontracture and calcium oscillations [85]. Similar findings were reported by Matsuda and coworkers who demonstrated a pH-dependent elevation in intracellular calcium levels of reoxygenated cardiomyocytes which was attenuated by reoxygenation with acidic medium or treatment with amiloride, two approaches which would reduce NHE activity [86]. In addition to the scenario involving calcium overloading via Na–Ca exchange, it has also been proposed that a rapid NHE-dependent recovery in intracellular pH after reperfusion results in both mechanical and energetic abnormalities which may contribute to dysfunction associated with myocardial stunning of the reperfused myocardium and which are corrected by NHE inhibition [87].

7.1. Role of NHE in mediating toxic effects of ischemic metabolites

Emerging evidence suggests that NHE inhibition may also afford protection against the direct effects of various

cardiotoxic agents as well as the ability of some of these agents to increase ischemic and reperfusion injury. For example, in earlier studies we showed that NHE inhibitors can effectively reverse the ability of exogenous lactic acid to inhibit recovery after reperfusion of the ischemic myocardium [88]. Similarly, NHE inhibition prevents the ability of endothelin-1 to attenuate ventricular recovery after reperfusion [89]. Direct toxicity produced by various compounds may also be NHE-dependent. For example, the toxic effects of lysophosphatidylcholine (LPC), at least at low concentrations, can be markedly attenuated by both HOE 642 and methylisobutylamiloride in terms of reduction in cardiodepression, contracture development, disturbances in energy metabolism and ultrastructural damage [90]. The most profound protective effects were against a LPC concentration of 3 μM where almost complete inhibition of toxicity was observed, with less protection against 5 μM LPC. Both NHE inhibitors also protect against hydrogen peroxide-induced cardiac injury but have no effect against the deleterious effect of a superoxide anion generating system [91]. These findings suggest that NHE inhibition confers protection against metabolites produced during myocardial ischemia and that at least some of the salutary effects of these inhibitors in the ischemic and reperfused heart may be due to these properties, Moreover, these multifaceted protective effects of NHE inhibitors may explain their superior protective properties on the ischemic and reperfused myocardium.

7.2. NHE and apoptosis

There is now increasing evidence that apoptosis or 'programmed cell death' is an important response of the myocardium to ischemia which is rapid, precedes cell necrosis and appears to contribute to the overall sequelae of cardiac injury (reviewed in [92]). The role of NHE in this response has not been extensively studied although some evidence suggests involvement of the antiporter. Gottlieb and coworkers [93] have shown that amiloride as well as ethylisopropylamiloride reduced apoptosis in metabolically inhibited rabbit cardiomyocytes although in that report the role of NHE was not clearly defined. We recently demonstrated that HOE 642 significantly attenuated the development of early apoptosis in hearts subjected to 30 minutes of global ischemia with or without reperfusion [94]. Taken together, the results suggest, but do not necessarily confirm, a contributory role of NHE to this process. Clearly, further studies are required to delineate the contribution of the NHE to this potentially important process.

8. Role of NHE in postinfarction responses

Although not studied in great detail thus far, emerging evidence suggests that postinfarction responses may also be regulated by NHE. For example, Hasegawa et al. [95] showed that adding amiloride to the drinking water of rats with infarcted myocardium resulted in a significant attenuation of ventricular remodelling. Moreover, mice with dilated cardiomyopathy exhibited reduced cardiac hypertrophy associated with diminished angiotensin II levels when they were given oral amiloride for a 60 day period [96]. Although the mechanistic basis for this requires further studies and clarification and confirmation of these effects with more specific NHE inhibitors, when taken together the findings are attractive as they may suggest an important role of NHE in the chronic long-term response to infarction as well as potential for novel treatment modalities for cardiac hypertrophy and heart failure. It should be noted that with respect to the former, there is evidence that by virtue of increased glycolysis and proton production intracellular acidification is greater which could lead to stimulated NHE activity (reviewed in [97]). On the other hand, right ventricular hypertrophy in a ferret model revealed no changes in intrinsic NHE activity [98]. It has also been suggested that protein kinase C-dependent coupling of receptor-stimulated activation of NHE by either endothelin-1 or angiotensin II is impaired in the hypertrophic rat myocardium, although the relevance of this finding needs to be clarified [99].

9. Intracellular pH and potential role of NHE in ischemic preconditioning

The potential role, if any, of NHE in mediating myocardial ischemic preconditioning has not been elucidated although a number of investigators have examined this issue as well as the broader aspect of pH regulation in the preconditioned myocardium. Accordingly, it has been clearly documented that the protective effects of ischemic preconditioning is associated with diminished proton loading in the ischemic myocardium [100–104]. A potential basis for the reduced proton accumulation may be diminished proton production from glycolysis in the preconditioned myocardium [101,103]. The exact role of NHE in the proton regulation in the ischemic myocardium is, however, controversial. For example, it has been suggested that reduced proton accumulation in the preconditioned myocardium may reflect enhanced proton extrusion through the NHE since ethylisopropylamiloride inhibited the salutary effects of preconditioning [105]. These results would suggest a beneficial role of NHE, and conversely a deleterious effect of NHE inhibition with respect to myocardial preconditioning. However, other investigators found that NHE inhibition in the preconditioned heart, either with ethylisobutylamiloride [106] or HOE 642 [107], produced an additive protective effect compared to preconditioning alone, suggesting distinct and separate mechanisms of action of each approach towards cardioprotection. It has been clearly demonstrated that at concentrations which

effectively inhibited NHE, HOE 642 failed to reverse the preconditioning effect [107]. Moreover, Miura and colleagues [76] reported a lack of PKC involvement in the protective effect of NHE inhibition compared to the apparent requirement for PKC activation for bestowing preconditioning, thus providing further evidence for dissimilar mechanisms. It appears, therefore, that further studies are necessary in order to unravel this complex issue although when taken together the current evidence argues against a role of NHE in mediating the preconditioning response.

10. Clinical evaluation of HOE 642 (cariporide) in high risk cardiac patients

In the Spotlight Issue, Scholz et al. [108] demonstrated the protective effects of HOE 642, as well as its specificity for NHE-1 vis à vis other ion regulatory proteins. The fundamental work with this inhibitor has evolved into a currently ongoing clinical trial (GUARDIAN study) to evaluate HOE 642, under the non-proprietary name cariporide, in high risk patients with coronary artery disease. This international study which will last 18 months and involves 400 centers and 9000 patients, will determine the effect of various doses of cariporide in patients with acute coronary syndrome. The four major patient groups to be evaluated include (1) high-risk patients undergoing percutaneous transluminal coronary angioplasty, (2) high-risk patients undergoing coronary artery bypass graft, (3) patients with unstable angina and (4) patients with non-Q wave infarcts. Incidence of mortality and myocardial infarction represent the two primary endpoints. The development and realization of the GUARDIAN study reflect the impressive evidence, with virtually a complete absence of discordant results, which demonstrates the protective effects of NHE inhibitors in a wide variety of experimental models, as well as the rapid progress in determining the role of NHE in mediating the tissue injury that is bought about by myocardial ischemia and reperfusion.

11. Role of NHE in other forms of the diseased myocardium?

A potentially important avenue of future investigation concerns NHE regulation in long-term disease states. From a developmental point of view, a number of investigators [109,110] have reported that NHE in terms of mRNA expression as well as activity is reduced during postnatal development. Disease states, on the other hand, may be associated with either increased or decreased NHE. For example, increased NHE activity has been demonstrated in hearts from spontaneously hypertensive rats [111].

11.1. NHE in diabetic heart

Hearts from diabetic animals have been reported to have reduced NHE activity which may be the basis for increased resistance to ischemia and reperfusion (reviewed in [112]). It has been suggested that the reduced activity may be related to decreased intracellular calcium levels in myocytes from diabetic hearts, and that this would lead to reduced NHE phosphorylation by calmodulin-dependent protein kinase [113]. The diminished ability to extrude protons via NHE appears also to be important in the regulation of cardiac ion currents. For example, inhibition of the transient outward potassium current is greater in myocytes from diabetic hearts, compared to control, following acid loading [114].

12. Conclusions

Approximately three years have passed since the reviews and articles of the Spotlight Issue of *Cardiovascular Research* were written. In this time, there have been impressive developments on all fronts, from the regulation of the Na–H exchanger on the molecular level to its functioning on the cellular and organ level, under normal as well as pathological conditions. Clearly, in the field of cardiac therapeutics the excellent protection against ischemic injury afforded by NHE inhibitors is virtually unparalleled [115] and may serve to explain the rapid progress in this area including the establishment of clinical studies. While we have a better understanding of some aspects of NHE, we are still lacking insights in many more. On the molecular level, much work will be needed to identify the elements on the exchanger protein that are essential for its transport function and regulation. On the cellular level, the specific regulatory pathways that modulate NHE activity in the cardiac myocyte will need to be elucidated. On the systemic level, we have yet to understand the causal relationship between NHE activity and disease such as hypertension; when does an altered NHE activity contribute to the development of a disease state, and when is it merely a byproduct of the disease? All these are topics that are under active investigation. The next few years will tell us how these efforts will bring us closer to the answer to these questions.

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