Physiology and pathophysiology of Na\(^+/H^+\) exchange and Na\(^+-K^+-2\text{Cl}^-\) cotransport in the heart, brain, and blood

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Physiology and pathophysiology of Na\(^+/H^+\) exchange and Na\(^+-K^+-2\text{Cl}^-\) cotransport in the heart, brain, and blood. Am J Physiol Regul Integr Comp Physiol 291: R1–R25, 2006. First published February 16, 2006; doi:10.1152/ajpregu.00782.2005—Maintenance of a stable cell volume and intracellular pH is critical for normal cell function. Arguably, two of the most important ion transporters involved in these processes are the Na\(^+/H^+\) exchanger isoform 1 (NHE1) and Na\(^+-K^+-2\text{Cl}^-\) cotransporter isoform 1 (NKCC1). Both NHE1 and NKCC1 are stimulated by cell shrinkage and by numerous other stimuli, including a wide range of hormones and growth factors, and for NHE1, intracellular acidification. Both transporters can be important regulators of cell volume, yet their activity also, directly or indirectly, affects the intracellular concentrations of Na\(^+\), Ca\(^{2+}\), Cl\(^-\), K\(^+\), and H\(^+\). Conversely, when either transporter responds to a stimulus other than cell shrinkage and when the driving force is directed to promote Na\(^+\) entry, one consequence may be cell swelling. Thus stimulation of NHE1 and/or NKCC1 by a deviation from homeostasis of a given parameter may regulate that parameter at the expense of compromising others, a coupling that may contribute to irreversible cell damage in a number of pathophysiological conditions. This review addresses the roles of NHE1 and NKCC1 in the cellular responses to physiological and pathophysiological stress. The aim is to provide a comprehensive overview of the mechanisms and consequences of stress-induced stimulation of these transporters with focus on the heart, brain, and blood. The physiological stressors reviewed are metabolic/exercise stress, osmotic stress, and mechanical stress, conditions in which NHE1 and NKCC1 play important physiological roles. With respect to pathophysiology, the focus is on ischemia and severe hypoxia where the roles of NHE1 and NKCC1 have been widely studied yet remain controversial and incompletely elucidated.

ischemia; hypoxia; intracellular pH; intracellular sodium concentration

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1 INTRODUCTION. The Na\(^+/H^+\) exchanger isoform 1 (NHE1) and the Na\(^+-K^+-2\text{Cl}^-\) cotransporter isoform 1 (NKCC1) are ubiquitous, electroneutral Na\(^+\)-dependent transporters with major roles in regulation of cellular volume and intracellular ion concentrations. NHE1 and NKCC1 are major Na\(^+\) influx pathways in essentially all cell types studied and exhibit multiple similarities with respect to regulation and physiological roles, and, consequently, in the pathophysiological consequences of their inappropriate function. Yet there are also important differences in their roles in specific tissues and processes. Mounting evidence indicates that both transporters are central to the cell damage induced by ischemia/hypoxia in various organs and thus of substantial clinical interest. An integrated view of the relative roles of NHE1 and NKCC1 in the cellular response to physiological and pathophysiological stress is therefore of major importance in understanding the physiology and pathophysiology of these transporters. However, such analyses have so far not been available in the literature. The aim of this review is to provide a comprehensive comparative view of the mechanisms and consequences of physiological and pathophysiological stimulation of these transporters in the heart, brain, and blood. In each of these tissues, we will examine the general relevance of the following chain of events in cell damage: stimulation of NHE1 and/or NKCC1, increases in intracellular Na\(^+\) concentration ([Na\(^+\)]\text{\(_i\)}) and cell volume, Na\(^+\)/Ca\(^{2+}\) exchange-mediated increases in intracellular free Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\text{\(_i\)}), and finally, decreased ATP levels and altered intracellular pH (pH\(_i\)).

Maintenance of a low [Na\(^+\)]\text{\(_i\)} is critical for normal cell function. Under steady-state conditions, [Na\(^+\)]\text{\(_i\)} is generally maintained below 20 mM, whereas the extracellular Na\(^+\) concentration ([Na\(^+\)]\text{\(_e\)}) is \(~140\) mM. This sevenfold concentration difference, together with the negative membrane potential (V\(_m\)), constitutes a substantial inward driving force for Na\(^+\), which is used ubiquitously to drive a wide variety of transport processes.

Cell volume, and/or net intracellular osmolyte concentration plays a pivotal role in a wide range of cellular processes, and consequently must be tightly controlled to maintain normal cell function. Osmotic cell shrinkage, resulting in vivo from either a decrease in intracellular osmolarity or an increase in extracellular osmolarity (106), therefore, generally activates compensatory ion (and osmotically obliged water) uptake, which persists until volume recovery is complete. The most important acutely shrinkage-activated ion transport mechanisms are I)
NHE1 functioning in parallel with a Cl⁻/HCO₃⁻ exchanger (AE), and 2) NKCC1 (106). In most cells, the activity of these transporters leads to a full or partial volume recovery, also known as the process of regulatory volume increase (RVI). Regulation of volume by NHE1 and/or NKCC1 after cell shrinkage can be associated with an incipient increase in [Na⁺], and stimulation of NHE1 or NKCC1 in nonshrunken cells by, e.g., hormones and growth factors, will result in both water and Na⁺ uptake. Yet in healthy cells, [Na⁺], is maintained relatively constant because of the activity of the Na⁺-K⁺-ATPase.

Another parameter fundamental to cell function and requiring tight homeostasis is intracellular pH (pHᵢ) (28, 48). In most mammalian cells, steady-state pHᵢ is maintained in the range of 7.0–7.2, although this may vary widely (28, 48). Even though extracellular pH (pHₑ) is ~7.4, given the negative Vᵢ, there is a substantial net inward electrochemical driving force for H⁺ per se. Additionally, metabolic processes create a net cellular H⁺ load, and H⁺ must be actively extruded from the cytoplasm. In most cases, acid-stimulated, H⁺ extruding transporters utilize the inward Na⁺ gradient, the most important players being Na⁺/H⁺ exchange, Na⁺-coupled Cl⁻/HCO₃⁻ exchange, and Na⁺-HCO₃⁻ cotransport (28). Thus regulation of pHᵢ after an acid load may increase both [Na⁺] and cell volume. On the other hand, stimulation of NHE1 or NKCC1 may lead to changes in pHᵢ, the magnitude of this effect being dependent on the activity of AE; NHE1 is functionally coupled to AE at a ratio of ~1 by changes in pHᵢ, and therefore [HCO₃⁻], (for a discussion, see Ref. 40). At 1:1 coupling, HCO₃⁻ influx via AE perfectly buffers H⁺ efflux via NHE1, and NHE1 activity is without effect on pHᵢ. However, when the activity of NHE1 exceeds that of AE, intracellular alkalinization results (see Ref. 40). Although NKCC1 activity per se is without effect on pHᵢ, it may be noted that this transporter may be indirectly coupled to pHᵢ changes through [Cl⁻], that is, an increase in [Cl⁻], causes AE to operate in the direction of recycling Cl⁻ out of the cell, resulting in HCO₃⁻ influx and an intracellular alkalinization in cells with a robust AE.

Furthermore, because of the interplay of these transporters with AE and the Na⁺-K⁺-ATPase, stimulation of NHE1, as well as of NKCC1, can affect [Cl⁻] and [K⁺], both essential modulators of multiple cell functions from effects on Vᵢ and excitability to cell death and proliferation (283, 342). With respect to cations, the eventual result of both NHE1 and NKCC1 stimulation under conditions of optimal Na⁺-K⁺-ATPase activity is increased [K⁺], rather than increased [Na⁺], (see Ref. 40). However, as will be discussed below, Na⁺-K⁺-ATPase activity is frequently compromised in stressed cells, such that both NHE1 and NKCC1 can elicit sizable increases in [Na⁺]. Finally, whereas either NHE1 coupled with AE, or NKCC1 by itself, can increase [Cl⁻], it should be noted that functional coupling of AE with these transporters may have opposite effects on [Cl⁻], i.e., 1:1 coupling of NHE1 and AE will tend to increase [Cl⁻], whereas 1:1 coupling of NKCC1 with AE is more likely to limit increases in [Cl⁻].

The overall aim of this review is to provide the reader with the knowledge required to appreciate the respective roles of NHE1 and NKCC1 in the detrimental events resulting from ischemia and severe hypoxia in the heart, brain, and blood. Thus we will first (section 2) provide an update on the defining characteristics of these two transporters. This will be followed (section 3) by an overview and comparative analysis of the roles of NHE1 and NKCC1 in the response to a variety of physiological stressors and, finally, (section 4) by an in-depth description of the involvement of NHE1 and NKCC1 in ischemia and severe hypoxia in the heart, brain, and blood, respectively. A brief overview (section 5) of the relatively scarce evidence regarding the roles of NHE1 and NKCC1 in apoptosis and necrosis in ischemia/hypoxia concludes the review.

2 NHE1 and NKCC1: Defining Characteristics

2.1 NHE1

2.1.1 Tissue and cellular localization. NHE1 (SLC9A1, Fig. 1A) belongs to the family of Na⁺/H⁺ exchangers of which nine are identified to date (195, 209). NHE1 is expressed in virtually all cell types studied (209, 222, 238) with avian red blood cells (RBCs) as a notable exception in that they lack volume-sensitive Na⁺/H⁺ exchange while exhibiting robust NKCC1 activity (140). In polarized cells, NHE1 is generally located in the basolateral (abluminal) membrane (see Ref. 209). In the
microvascular endothelial cells of the blood-brain barrier (BBB), NHE1 appears to contribute to secretion of Na\(^{+}\), Cl\(^{-}\), and water into the brain during ischemia (as discussed in section 4); however, it is as yet unknown whether NHE1 localizes to the luminal or abluminal membrane of the BBB cells (68, 307). At least in some cells, NHE1 localizes to discrete plasma membrane subdomains, examples being the preferential localization to focal adhesions in fibroblasts (97) and to intercalated discs and transverse tubules in cardiomyocytes (227).

2.1.2 Energetics, kinetics, and control. The driving force for Na\(^{+}/H^{+}\) exchange via NHE1 is \(\Delta G = \Delta V + \Delta F\), where \(\Delta V\) is the free energy change due to the ionic potential difference between the two compartments, \(\Delta F\) is the free energy change due to the chemical potential difference, and \(\Delta G\) is the free energy change.

The NH\(_2\)-terminal domain with the 12 TM regions (~500 amino acids) is highly conserved between vertebrate species and is responsible and sufficient for ion translocation by NHE1 (319). The COOH-terminal cytoplasmic domain (~300 amino acids) is the main site of regulation of NHE1 function and exhibits a considerable sequence variation between species. This COOH-terminal domain contains binding sites for a large number of ancillary proteins and lipid mediators, primarily in the region closest to the TM domains (Fig. 1A). Although not all of these will be further discussed in this review, it should be mentioned that NHE1 binding partners shown to interact with the COOH-terminal domain include the plasma membrane-cytoskeleton linker ezrin [of the ezrin/radixin/moesin (ERM) protein family], calcineurin homolog protein (CHP), the ste20-related kinase Nck-interacting kinase, protein phosphatase 1 (PP1), calmodulin, carbonic anhydrase, and the phospholipid phosphatidyl-inositol(4,5)bisphosphate. The most COOH-terminal part of this region contains multiple protein kinase consensus sites, the roles of which in NHE1 regulation have been extensively studied (see Refs. 181, 209, 222, and 238).

2.1.4 General physiological roles. By far the best characterized physiological functions of NHE1 are the regulation of cellular volume and pH, although other roles have also been described (see Refs. 209, 221, and 238). After cell shrinkage, rapid NHE1-mediated Na\(^{+}\) influx in conjunction with Cl\(^{-}\) influx via the AE restores cell volume, while after intracellular acidification, the NHE1-mediated H\(^{+}\) efflux efficiently restores pH\(_{i}\) (see, e.g., Refs. 209 and 222). Under normal physiological conditions, i.e., as long as such deviations from steady state are moderate and transient, this has the effect of normalizing cell volume or pH\(_{i}\) whereas after dramatic or prolonged deviations from steady-state pH\(_{i}\) or volume, NHE1-mediated effects on [Na\(^{+}\)] and pH\(_{i}\) are important in the cellular damage associated with such conditions, as will be discussed below.

An important aspect of NHE1 regulation is the effect of interactions between different stimuli. In Amphiiuma tridactylum RBCs, when stimulated by intracellular acidification, NHE1 remained active until pH\(_{i}\) reached its set point, and was not turned off by the cell swelling resulting from NHE1 activity (40). Conversely, when stimulated by cell shrinkage, NHE1 remained active until cell volume was restored, in spite of a substantial increase in pH\(_{i}\) (40), which, if acting alone, would otherwise deactivate NHE1. Similarly, a wide range of cell culture studies show that after stimuli, such as hormones, growth factors, and inhibitors of ser/ser phosphatases, which activate NHE1 at normal pH\(_{i}\) and volume, the exchanger can remain active in spite of substantially increased cell volume, pH\(_{i}\) and [Na\(^{+}\)] (244–226, 260, 318). Such increases in cell volume and pH\(_{i}\) may modulate, e.g., cell proliferation (see Ref. 221). This has led to the proposal that increased NHE1 activity is a prerequisite for tumor cell proliferation, a notion which has, however, been disputed by other workers in the field (for a discussion, see Refs. 221 and 274). Again, a detailed discussion of this is beyond the scope of this review, but it should be noted that in recent years, the physiological roles of NHE1 have been suggested to include modulation of, e.g., cell morphology, migration, and invasion (see Refs. 209 and 238). Interestingly, these roles of NHE1 appear in some cases to be at least partly unrelated to ion transport, and rather involving direct interactions of NHE1 with, e.g., ERM proteins (see Ref. 238).
Consistent with the fundamental roles of NHE1 in normal cell function, NHE1 knockout mice exhibit multiple abnormalities, including locomotor problems, growth retardation, abnormal membrane excitability, and Na⁺ permeability of hippocampal CA1 neurons, and 70% of the knockout animals die before weaning (20, 99). On the other hand, these mice exhibit significant protection from ischemic damage in both the heart and the brain (section 4).

2.2 NKCC1

2.2.1 Tissue and cellular localization. The Na⁺-K⁺-2Cl⁻ cotransporter NKCC1 (SLC12a2, also known as BSC2; Fig. 1B) is expressed in the great majority of cell types studied, whereas the other cloned isoform, NKCC2 (SLC12a1, BSC1), is restricted to the kidney (see Ref. 254). RBCs of certain species, such as teleost fishes and amphibians, however, do not appear to exhibit volume-sensitive Na⁺-K⁺-2Cl⁻ cotransport, yet exhibit robust NHE activity (37, 108, 141). NKCC1 is found on the basolateral membrane of secretory epithelial cells (254). An exception to this is the microvascular endothelial cells of the BBB, which secrete Na⁺ and Cl⁻ from the blood into the brain. In this case, NKCC1 is located in the apical (luminal) membrane (205) and secretion (i.e., transport from blood into tissue) occurs in an apical-to-basolateral direction. Also, in the choroid plexus, NKCC1 appears to be located in the apical membrane (235).

2.2.2 Energetics, kinetics, and control. The driving force for Na⁺-K⁺-2Cl⁻ cotransport via NKCC1 is \( \Delta \mu \text{Na}^+ + \Delta \mu \text{K}^+ + 2 \Delta \mu \text{Cl}^- = RT \ln \left(\frac{[\text{Na}^+]_o}{[\text{Na}^+]_i}\right) + RT \ln \left(\frac{[\text{K}^+]_o}{[\text{K}^+]_i}\right) + 2RT \ln \left(\frac{[\text{Cl}^-]_o}{[\text{Cl}^-]_i}\right) = RT \ln \left(\frac{[\text{Na}^+]_o[\text{K}^+]_o[\text{Cl}^-]_o}{[\text{Na}^+]_i[\text{K}^+]_i[\text{Cl}^-]_i}\right) = RT \ln \left(\frac{V_m}{V_n}\right) < 0 \), where \( V_m \) is the membrane potential and \( V_n \) is the equilibrium potential of Cl⁻ (5, 95). Moreover, astrocytes from NKCC1 knockout mice exhibited reduced basal [Na⁺]ᵢ, suggesting a role for NKCC1 in regulating basal astrocyte [Na⁺] (254). Vectorial NaCl transport in secretory epithelia is dependent on the functional coupling of the basolaterally located NKCC1 to apical Cl⁻ channels, basolateral K⁺ channels, and the Na⁺-K⁺-2Cl⁻-ATPase (e.g., Ref. 95). In the BBB, NKCC1 may function in vectorial transport of NaCl from blood into brain, and as discussed below, increased NKCC1 activity in the BBB contributes to cerebral edema formation in ischemia (81, 203, 205). A role for NKCC1 in regulation of cell proliferation has also been proposed, although this is less well established (see Ref. 254). Consistent with the proposed major physiological roles of NKCC1, the phenotype of NKCC1 knockout mice includes deafness, imbalance (Shaker/Waltzer phenotype), and decreased Cl⁻ secretion in e.g., intestine and trachea (60, 77). However, as will be discussed in section 4, they also exhibit less brain damage in experimental stroke (47).

3 NHE1 and NKCC1 in the Response to Physiological Stress

As described above, NHE1 and NKCC1 play important roles in reestablishing homeostasis after physiological perturbations to cell volume, pHᵢ, and [Cl⁻], and in the adaptive responses to a variety of physiological stressors. We define physiological stress in broad terms as the stimuli to which cells are exposed under normal physiological conditions, resulting in temporary deviations from the steady state. The relevant physiological stressors are obviously numerous and will depend on the species and cell type, but for the purpose of the present review, we focus on three major categories: metabolic and exercise stress, osmotic stress, and mechanical stress. All of these conditions, if sufficiently severe, may of course become patho-
physiological; for instance, the ability of severe hypertonic stress to elicit cell death is well established (29, 36, 201).

Metabolic stress in the form of a reduced cellular ATP level may result from, e.g., exercise stress, mild hypoxia, or reduced availability of nutrients, and is typically associated with reduced pH, as well as with changes in the cellular level of reactive oxygen species (ROS). Exercise stress exhibits several similarities with hypoxic stress at the level of plasma hormone and ion concentrations. In mammals, vigorous exercise is associated with reductions in microvascular oxygen pressure and ion concentrations. In mammals, vigorous exercise is similar to hypoxic stress at the level of plasma hormone and ion concentrations. In mammals, vigorous exercise is similar to hypoxic stress at the level of plasma hormone and ion concentrations.

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Table 1. Localization and physiological roles of NHE1 in heart, brain, and blood

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Localization</th>
<th>Proposed physiological roles</th>
<th>References</th>
</tr>
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<tbody>
<tr>
<td>Heart</td>
<td>Cardiomyocytes (t-tubules and intercalated discs)</td>
<td>pH regulation (metabolic acid extrusion)</td>
<td>(80, 183, 227, 325, 333)</td>
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<tr>
<td></td>
<td>Aortic endothelial cells</td>
<td>Cell stretch response</td>
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<td></td>
<td></td>
<td>Cell volume regulation?</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td>pH regulation</td>
<td>(18, 100)</td>
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<tr>
<td>Brain</td>
<td>Neurons (all types studied)</td>
<td>pH regulation (metabolic acid extrusion)</td>
<td>(212, 223)</td>
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<tr>
<td></td>
<td>Gial cells (all types studied)</td>
<td>pH regulation (metabolic acid extrusion)</td>
<td>(47, 273)</td>
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<tr>
<td></td>
<td>Brain microvascular endothelial cells</td>
<td>Cell volume regulation</td>
<td></td>
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<tr>
<td></td>
<td>Choroid plexus epithelial cells</td>
<td>Vectorial Na+ transport pH regulation</td>
<td>(68, 112, 281, 307)</td>
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<tr>
<td>Blood</td>
<td>Red blood cells</td>
<td>Regulation of hemoglobin O2 affinity and saturation (lower vertebrates)</td>
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<tr>
<td></td>
<td>Platelets</td>
<td>Cell volume regulation</td>
<td></td>
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<tr>
<td></td>
<td>Leukocytes</td>
<td>pH regulation</td>
<td>(127)</td>
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<tr>
<td></td>
<td></td>
<td>Cell volume regulation, pH regulation, activation</td>
<td>(37, 40, 123, 216)</td>
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</table>

The heart depends primarily on aerobic metabolism under physiological conditions, yet ATP produced through the glycolytic pathway appears to be necessary for some cardiac functions, including Na+ homeostasis (42). The proportion of ATP produced glycolytically is further increased during exercise and other physiological conditions of increased energy demand, leading to an increased requirement for H+ extrusion (42, 92). Cell volume perturbations in the heart occur predominantly under pathophysiological conditions, such as diabetic coma, septic shock, or ischemia (327). Hypertonic cell shrinkage negatively affects cardiac contractility, and hypotonic swelling shortens the cardiac action potential (110, 327). In spite of these severe consequences of cardiomyocyte volume perturbations, the extent to which cardiomyocytes are able to volume regulate is a controversial issue, with most studies concluding that these cells do not perform RVI (206, 327). Shrinkage-induced stimulation of both NHE1 and NKCC1 has, however, been reported in the heart, as will be discussed below.

3.1 Physiological Stress in the Heart

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3.1.1 Roles of NHE1. NHE1 appears to be the only, or by far, the predominant NHE isoform in cardiac myocytes (80, 210, 310), where it has been shown to localize mostly to the transverse tubules and intercalated disc regions (227). Consistent with the high demand for metabolic acid extrusion under the heart's high ATP utilization rate, NHE1 has been shown to be the predominant NHE isoform present in cardiac myocytes (80, 210, 310) and has been shown to be the predominant NHE isoform present in cardiac myocytes (80, 210, 310).
steady-state conditions in the heart, the myocardial NHE1 exhibits high tonic activity and plays a major role in cardiac pH homeostasis under physiological conditions (80). In congruence with the proposed importance of glycolytically derived ATP for cardiac Na\(^+\) homeostasis (42), NHE1 has been proposed to be specifically dependent on ATP produced by glycolysis rather than by oxidative phosphorylation (291). This linkage has interesting implications, because NHE1 stimulation may be preferentially coupled to ATP produced under conditions (i.e., increased glycolysis) that necessitate increased H\(^+\) extrusion.

As in most cell types, NHE1 is also stimulated by hypertonic stress in both mature and neonatal cardiac myocytes (183, 325). This may, however, not lead to an RVI response, as it has been suggested that mature cardiomyocytes cannot perform RVI (327).

NHE1 plays an important role in the physiological events elicited in the heart by mechanical stress in the form of cell stretch. In isolated rabbit myocardium, the slow force response component of the muscle stretch response was dependent on stretch-induced NHE1 activation, followed by increased cytosolic [Na\(^+\)] and increased [Ca\(^{2+}\)] because of Na\(^+\)/Ca\(^{2+}\) exchanger (NCX) reversal (316). Similar findings have been reported in other cardiomyocyte preparations (333). Interestingly, stimulation of NHE1 by cardiomyocyte stretch has been found to be upstream of stretch-induced Raf-1 and MAPK activation and subsequent hypertrophy (299, 333).

### 3.1.2 Roles of NKCC1

Evidence for the presence of NKCC1 in whole mammalian hearts, as well as in isolated ventricular myocytes, has been obtained both at the functional level (7, 65, 252) and at the mRNA and protein level (6, 241, 314). NKCC1 has also been characterized in aortic endothelial cells (341). Compared with the abundant literature on NHE1, relatively little is known about the roles and regulation of NKCC1 in the heart. NKCC1 likely plays a role in maintaining cardiac resting [Na\(^+\)], and [Cl\(^-\)], (84, 114), yet we are not aware of direct studies of the effects of physiological metabolic stress on NKCC1 in the heart.

A role for NKCC1 in isotonic and anisotonic cell volume regulation has been proposed in rabbit ventricular myocytes (65) and in smooth muscle cells from rat aorta (208). Yet, others failed to find detectable NKCC1 activity after hypertonic stress in the heart (71). NKCC1 is also stimulated by osmotic shrinkage of aortic endothelial cells in which RVI was inhibited by bumetanide (202). The RVI was, however, not complete by 30 min, suggesting that complete RVI is a slow process or that the cells are unable to completely recover (202). In other studies, an RVI response could not be demonstrated in aortic endothelial cells (see Ref. 206). Finally, in bovine aortic endothelial cells, mechanical stress in the form of steady laminar shear stress activated and upregulated NKCC1, an effect that was shown to involve shear stress-induced activation of K\(^+\) and Cl\(^-\) channels (296).

### 3.2 Physiological Stress in the Brain

In the brain, metabolic stress requiring regulation of pH\(_i\) occurs under physiological conditions, because both neurons and glial cells undergo significant shifts in pH\(_i\) during increased neuronal activity. For instance, in cultured hippocampal neurons (322) and brain stem slices (308), depolarizing stimuli were shown to elicit intracellular acidification. This decrease in pH\(_i\) is, at least in part, dependent on Ca\(^{2+}\) entry and in vertebrate neurons appears to mainly reflect metabolic acid production (322, 344). Additionally, because of its permeability to HCO\(_3^-\), activation of the GABA\(_A\) receptor also decreases pH\(_i\), as shown in both cultured astrocytes (126) and acutely isolated hippocampal neurons (219).

Under physiological conditions, the volume of both neurons and glial cells is constantly challenged by the transmembrane ion movements occurring during normal neuronal activity, and these volume changes may, in turn, affect brain function (163, 218, 283). Brain volume regulation, associated with net cellular uptake of Na\(^+\), K\(^+\), Cl\(^-\), and water, and a concomitant reduction in extracellular volume, has been measured in vivo in rats exposed to acute hypernatremia (54). Glial cells are capable of cell volume regulation after acute osmotic swelling and shrinkage (69, 175). With respect to neurons, this is more controversial; in several neuronal preparations, no volume regulation could be detected after acute osmotic stress (2, 11), and it appears that more severe (nonphysiological) osmotic challenges are required for neurons to be capable of acute RVI (see e.g., Ref. 53). On the other hand, immature cortical

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**Invited Review**

**ION TRANSPORT, CELL VOLUME, AND STRESS**

### Table 2. Localization and physiological roles of NKCC1 in heart, brain, and blood

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<tr>
<th>Tissue</th>
<th>Localization</th>
<th>Proposed physiological roles</th>
<th>References</th>
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<tr>
<td>Heart</td>
<td>Cardiomyocytes</td>
<td>Cell volume regulation?</td>
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<td>Regulation of basal [Na(^+)], and [Cl(^-)]</td>
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<td>Cell volume regulation?</td>
<td>(202, 206, 296, 341)</td>
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<td>Responses to shear stress</td>
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<td>Aortic endothelial</td>
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<td>Regulation of basal [Na(^+)], GABAergic signaling</td>
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<td>Cell volume regulation?</td>
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<td>Regulation of plasma [K(^+)]?</td>
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<td>Vectorial ion transport</td>
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<td>Red blood cells</td>
<td>Regulation of plasma [K(^+)]?</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Cell volume regulation?</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Leukocytes</td>
<td>Regulation of MAPK activity and proliferation</td>
<td>(214)</td>
</tr>
</tbody>
</table>

[Na\(^+\)], intracellular Na\(^+\) concentration; [Cl\(^-\)], intracellular Cl\(^-\) concentration; [K\(^+\)], K\(^+\) concentration.
neurons exhibited RVI after a relatively modest increase in extracellular osmolarity (265).

3.2.1 Roles of NHE1. NHE1 is widely expressed in the central nervous system (CNS) and is the most abundant NHE isoform in the cerebral cortex (162, 210). Na\(^+\)/H\(^+\) exchange activity has been found in virtually all neuronal and glial cell types studied, although it was not always unequivocally established whether the isoform in question was NHE1 (48). This is an important question, since NHE2, NHE3, NHE4, and NHE5 are also found in the brain, although with more restricted patterns of distribution (48). NHE1 message has also been detected in choroid plexus (127; Praetorius J, personal communication), which is known to exhibit amiloride-sensitive Na\(^+\)/H\(^+\) exchange (191). Finally, message for NHE1 (and also for NHE2, NHE3, and NHE4) was detected in BBB endothelial cells (68, 127, 281, 315), and there is evidence that NHE1 is an important mediator of steady-state Na\(^+\) transport across the BBB (68).

NHE1 plays a key role in pH\(_r\) regulation in both neurons and glial cells under steady-state conditions and after intracellular acidification (48, 162, 173, 212, 223). Recovery of pH\(_r\) after metabolic acid production associated with neuronal activity (see section 3.2) appears to be a major function of NHE1 in brain neurons. Consistent with this view, both acutely dissociated CA1 pyramidal neurons from NHE1 null mice (339) and astrocytes from NHE1 null mice (137) exhibit reduced steady-state pH\(_r\), as well as reduced or virtually absent H\(^+\) extrusion after intracellular acidification. Shrinkage-induced NHE1 stimulation has been demonstrated in primary rat astrocytes (273). No studies have, to our knowledge, directly demonstrated osmotically induced NHE1 activity in neurons, neither in the intact brain nor in culture.

3.2.2 Roles of NKCC1. NKCC1 mRNA has been detected in choroid plexus, cerebellum, brainstem, and to a lesser extent, cerebral cortex and hypothalamus (128, 235), reflecting NKCC1 expression in a wide range of neuronal cell types (129, 235), in glial cells (oligodendrocytes and astrocytes) in various regions of the brain (128, 150, 205, 323, 335), and in cerebral microvascular endothelial cells (203, 205, 292, 293). In the latter, functional and immunocytochemical evidence indicated the presence of NKCC1 in the luminal membrane (202, 205), although others have failed to detect brain microvessel NKCC1 mRNA in situ hybridization studies (235). Interestingly, in the choroid plexus epithelium, NKCC1 is extremely abundant and is only expressed on the apical membrane (235).

The main physiological roles of NKCC1 in the brain appear to be 1) the regulation of [Cl\(^-\)], and thus, γ-aminobutyric (GABA)-ergic signaling (GABA being excitatory at high [Cl\(^-\)], and inhibitory at low [Cl\(^-\)]), (59, 295); 2) ion transport across the BBB (81, 203, 205) and choroid plexus (330); and 3) although this is less well established, in cell volume regulation (see below). A role in regulating basal [Na\(^+\)] has also been suggested, based on studies of NKCC1\(^{-/-}\) astrocytes (289).

NKCC1 expression is high in immature neurons and decreases with maturation, whereas the inverse is true for the K\(^+\)-Cl\(^-\) cotransporter KCC2 (see Ref. 59). These changes are thought to underlie the decrease in neuronal [Cl\(^-\)], during development and the shift from excitatory to inhibitory GABA signaling (see Ref. 59). NKCC1 still contributes to regulation of [Cl\(^-\)] in adult neurons, some of which, including, e.g., dorsal root ganglion neurons, exhibit a high NKCC1 expression, high [Cl\(^-\)], and depolarizing responses to GABA (see Ref. 59).

In neurons capable of cell volume regulation after osmotic shrinkage, NKCC1 may play a role in this process. In immature cortical neurons, NKCC1 was activated by osmotic shrinkage, and a bumetanide-sensitive RVI was demonstrated (265). Osmiotically induced Na\(^+\)-K\(^+\)-2Cl\(^-\) cotransport was also observed in squid giant axons (32) and in PC12 cells (151). In C6 glioma cells, NKCC1 appears to be important in both isotonic volume homeostasis and RVI (45, 175). Similarly, RVI was robust in wild-type (NKCC1\(^{+/+}\)) astrocytes, but absent in NKCC1\(^{-/-}\) astrocytes (289). NKCC1 is also stimulated by osmotic shrinkage in cerebral microvascular endothelial cells, although it is not yet known whether this translates into an RVI response in these cells (202).

3.3 Physiological Stress in the Blood

Exercise stress increases [K\(^+\)], [Na\(^+\)], and [Cl\(^-\)] in human RBCs (153, 172), and as will be discussed below, in some species, RBCs may play a role in buffering plasma K\(^+\), at least during high-intensity exercise (153). RBCs from teleost fishes have been extensively used as models for mild hypoxic stress, given the often extremely high exercise level of these species, frequent exposure to hypoxic conditions, and unique requirement for adaptation to large changes in Po\(_2\), as well as the fact that teleost RBCs are nucleated cells with protein synthesis, mitochondria, etc., resembling those of mammalian non-RBC cells (e.g., Refs. 49 and 198). One general finding, pioneered in studies in RBCs from teleosts and other lower vertebrates, and confirmed in some mammalian RBCs, is that a number of ion transporters including, yet not limited to, NHE1 and NKCC1, exhibit marked O\(_2\) dependence (64; see Ref. 89). A role for hemoglobin (Hb) as an O\(_2\) sensor was suggested (89, 186); however, findings in trout hepatocytes indicate that this phenomenon is not limited to RBCs (311). A link with cell volume is suggested in that swelling-activated transporters are generally found to be stimulated, and shrinkage-activated transporters to be inhibited, by a decrease in O\(_2\) pressure (Po\(_2\)) (see Ref. 89).

3.3.1 Roles of NHE1. NHE1 appears to be the only NHE isofrom in mammalian RBCs (e.g., Ref. 259). Although NHE1 transport capacity decreases with RBC maturation (41) and is modest in most mammalian RBCs, including those of humans, NHE1 is present and functional in mature RBCs from many mammals, including dogs (216), rabbits (123), and humans (30). Moreover, many nucleated RBCs, such as those of teleosts (167, 225) and amphibians (174), exhibit robust NHE1 activity. In RBCs from both mammals and lower vertebrates, NHE1 is activated by acidic pH\(_r\), as well as by osmotic shrinkage (37, 123, 269). While RBCs are the focus here, it should be noted that NHE1 is also the predominant or only NHE isofrom in platelets and at least some types of leukocytes, in which it has been assigned important physiological roles (85, 96, 247).
Exercise stress elicits an increase in circulating catecholamines in teleosts, resulting in stimulation of NHE1, and, consequently, increased RBC pH, and cell volume (159), similar to what occurs in hypoxia (see section 4.1) or after direct β-adrenergic stimulation (225; see 222). In exercise-stressed human RBCs, increased Na+/Li+ countertransport following exercise stress has been described (1); however, the possible identity of this transporter with NHE1 is controversial (207).

In RBCs of teleosts and some amphibians, NHE1/β-NHE is stimulated under hypoxic conditions, by norepinephrine (NE) via β-adrenergic receptors (β-ARs), via decreased pH, and via a still poorly understood deoxygenation effect on the RBC NHE1 (see Refs. 89 and 222). The physiological consequence is that in response to hypoxic or exercise stress, the O₂ affinity of Hb is increased because of a combination of the NHE-mediated limitation of intracellular acidification, and dilution of Hb resulting from cell swelling (see Ref. 222). To the knowledge of the authors, no such studies exist for NHE1 in higher vertebrates, which, as discussed by Nikinmaa (197), generally have Hbs with lower Bohr/Haldane effects and employ different strategies for optimizing RBC O₂ transport properties.

Osmotic stress potently stimulates NHE1 in RBCs from a wide variety of species, with the interesting exception of some teleosts (see Ref. 222). The first demonstration of the role of Na⁺/H⁺ exchange functionally coupled to Cl⁻/HCO₃⁻ exchange in RVI came from studies of A. tridactylum RBCs in our laboratory (106, 222). This is now known to be a general strategy for RVI in a wide variety of cell types across the vertebrate phylum (106, 222), and the Amphiuma Na⁺/H⁺ exchanger has been cloned and found to be an NHE1 with high homology to mammalian NHE1s (174).

### 3.3.2 Roles of NKCC1

There is functional evidence for the presence of NKCC1 in RBCs from birds (161), as well as from mammals, including humans (79, 153, 165). Similar to NHE1 in teleost RBCs, NKCC1 in avian RBCs is stimulated by hypoxia and metabolic stress, by virtue of its potent stimulation both by β-adrenergic stimuli and by deoxygenation per se (161, 192). As discussed by Gibson et al. (89), the driving force for inward transport via NKCC1 in RBCs is markedly increased by even slight elevations of plasma [K⁺], and a role for NKCC1 as a dynamic buffer for plasma [K⁺] is conceivable. This may play a role in birds (158), which exhibit robust NKCC1 activity and experience extreme exercise stress during migratory flight, but also in mammals with high hematocrits, such as the horse (see Ref. 89). In humans, RBC K⁺ content is also elevated during exercise; however, this appears to be primarily mediated by the Na⁺-K⁺-ATPase, with little, if any, contributions from NKCC1, except under conditions when the Na⁺-K⁺-ATPase is inhibited (153). NKCC1 is also activated by osmotic cell shrinkage in both avian (161) and mammalian RBCs (165). It is interesting to note that as rat RBCs mature, the net driving force for NKCC1 changes direction, being inward in reticulocytes, yet outward in mature RBCs. This net outward transport by NKCC1 may under some conditions contribute to reducing the isotonic volume of the RBCs (166; see also Refs. 158 and 165).

Finally, it may be noted that NKCC1 has also been found in leukocytes, in which it has been proposed to play a role in regulation of MAPK activity and proliferation (214).

### 4 NHE1 and NKCC1 in Pathophysiological Stress: Ischemia and Severe Hypoxia

#### 4.1 Ischemia and Severe Hypoxia in the Heart

#### 4.1.1 General Aspects

- **Energy status and ion homeostasis:** Ischemia is associated with a marked decrease in the cellular ATP level in the heart (see Ref. 42). It should be noted that the reported rates of decline of ATP levels in ischemic perfused hearts vary considerably, from about one-third reduction within 45 min in ferret hearts (66) to about 50% reduction within 15–20 min in rat hearts (243, 298). Such differences in ATP levels are likely to be due, at least in part, to differences in experimental protocol (see Ref. 4) and may well account for some of the controversy discussed below regarding transporter regulation (sections 4.1.2 and 4.1.3).

- **The Na⁺-K⁺-ATPase has generally been assumed to be inhibited in ischemia because of ATP-depletion, a notion that has been confirmed in several models of ischemia in the heart (52, 98, 285). Consequently, the ischemia-induced increase in [Na⁺], in the heart was originally thought to reflect inhibition of the Na⁺-K⁺-ATPase (98, 285). On the other hand, others have found no evidence of Na⁺-K⁺-ATPase inhibition in early ischemia (237), and more recent studies in perfused rabbit hearts have indicated that in early ischemia, Na⁺-K⁺-ATPase activity is actually increased rather than decreased, presumably as a consequence of the increases in [Na⁺] and [K⁺], (7). As will be detailed below, the ischemia-induced increases in [Na⁺], and [Ca²⁺], (10, 271; see also Refs. 189 and 284) are now known to largely reflect stimulation of NHE1 and consequent uptake of Ca²⁺ via the NCX in response to increases in [Na⁺]. In agreement with the notion that NCX reversal/equilibrium plays an important role in the ischemia/reperfusion-mediated increase in [Ca²⁺], in the heart (268), NCX has been shown to be near or at thermodynamic equilibrium during ischemia (for a discussion, see Ref. 8), indicating that it is a major Ca²⁺ influx pathway under these conditions. Increases in [Cl⁻], during ischemia have also been demonstrated in the...
heart (242). Ischemia, moreover, elicits cardiac cell swelling, because of a combination of net inward ion transport and lactate production by anaerobic glycolysis (13, 327). Cardiac pH homeostasis is also severely compromised by ischemia. On exposure to ischemic conditions, pH in the heart reaches about 6–6.5, and pH falls rapidly, by about 0.5 pH unit in the first 5 min (155, 261, 286).

4.1.1.2 Signaling events. Myocardial ischemia activates the sympathetic nervous system and leads to local release of the catecholamine NE in the ischemic heart (267). In early cardiac ischemia and under normal physiological conditions, NE release is exocytotic and Ca2+ dependent, whereas in prolonged ischemia it is carrier mediated and reflects reversal of the driving force for the NE transporter (NET) (152). The renin-angiotensin system also is activated in ischemic hearts, resulting in increased local angiotensin (ANG II) formation. Consistent with a role for ANG II in ischemic damage, ANG II type 1 (AT1) receptor blockade reduces cardiac damage after ischemia, by mechanisms which are not fully elucidated (115, 122). Endothelin-1 (ET-1) contributes to myocardial damage in ischemia (see Refs. 133 and 297), and at least some of the cardiac effects of ANG II are mediated by ET-1 (185). Local thrombin levels are elevated in intracoronary thrombosis, the most frequent cause of acute myocardial ischemia (58). Finally, cytokines and inflammatory mediators play an important role in myocardial ischemia, in which neutrophil accumulation occurs, at least in part, as a result of increased IL-8 production and contributes to ischemic damage (82).

MAPKs, including extracellular signal-regulated kinase (ERK1/2) and the stress-activated kinases p38 MAPK and c-Jun NH2-terminal kinase (JNK) are important intracellular mediators after cardiac hypoxia and ischemia/reperfusion (12). There are some discrepancies as to whether these kinases are active in both ischemia and reperfusion. In some studies, ERK1/2 appears to be inhibited rather than activated in ischemia, and only activated on subsequent reperfusion (182; see Ref. 12). The ERK1/2 effector p90 ribosomal S kinase (p90RSK) has been found to be activated in the heart during both ischemia and reperfusion (182). Other classes of protein kinases are also implicated, for instance, there is evidence that at least some of the effects of G protein-coupled receptor agonists in cardiac ischemia, including catecholamines (acting via α1-ARs), ANG II, ET-1, and thrombin, are mediated via protein kinase C (PKC) (14).

Finally, reperfusion after ischemia/hypoxia is associated with release of ROS, for which a major role has been clearly established in the ensuing cell damage (91, 164). Notably, ROS are also released during hypoxia per se and may be an important signal in hypoxia sensing (102).

4.1.2 Mechanisms and Consequences of Stimulation of NHE1

From studies in isolated cardiac myocytes, perfused hearts, and in vivo models, it is widely acknowledged that NHE1 inhibitors exert protection against myocardial ischemic/hypoxic damage (138, 145, 155, 190, 286, 324). The essential role of NHE1 inhibition in cardiac protection was initially challenged by the fact that amiloride and amiloride derivatives also exert a varying inhibitory effect on Na+ channels (46) and on NCX (276). The finding that mice with an NHE1 null mutation are protected from myocardial ischemic damage (324) mitigates this concern. However, although a detailed description of this is beyond the scope of the present review, it should be kept in mind that noninactivating Na+ channels, Na+–HCO3− cotransport, in some cells NKCC1 (section 4.1.3), and at least in late ischemia Na+–K+–ATPase inhibition, also have been found to contribute to cardiac ischemic damage (52, 132, 176, 312).

A controversy has been whether NHE1 is active during the ischemic phase, and consequently whether NHE1 inhibitors need to be present throughout ischemia/reperfusion, or in the reperfusion phase only, to be effective (4, 189). Thus some studies have shown that although [Na+]i rose during the subsequent reperfusion phase, there was no or only a very small increase in [Na+]i during ischemia, suggesting that NHE1 is inhibited during this phase (182, 331). In contrast, numerous other studies have documented an immediate, robust increase in [Na+]i during ischemia and have concluded that NHE1 is active during both ischemia and reperfusion, although generally most active in the latter (155, 190, 232, 324; see also Ref. 189). Since pH rapidly falls to ~6–6.5 in ischemic hearts (see section 4.1.1.1), inhibition of NHE1 during the ischemic phase has been proposed to result from competition between Na+o and H+o for NHE1 binding (149). However, at the very acidic pHi which is prevalent in ischemic hearts, NHE1 is only modestly inhibited by acidic pHi (313; see also Ref. 4). NHE1 has a high effective half-maximal ATP concentration (2–5 mM) (43, 61) and has, accordingly, been found to be inhibited by the reduced ATP levels associated with metabolic inhibition (43, 329). Because the reported rate of decline of ATP levels in ischemic hearts varies considerably (see section 4.1.1.1), differences in ATP levels, in part, the result of differences in experimental protocol (see Ref. 4), undoubtedly account for some, if not all, of the controversy with regard to the activity of NHE1 during ischemia.

4.1.2.1 Mechanisms of stimulation of NHE1. Figure 2 summarizes major pathways of NHE1 regulation in ischemia/hypoxia. A major stimulus for maintaining high NHE1 activity during ischemia/reperfusion is the decreased pH. Consistent with this notion, the rate and pharmacological profile of Na+ uptake during hypoxia and during NH4Cl-induced acidification were similar in perfused rabbit hearts (10). In addition to pH, many other mechanisms have been reported to modulate cardiac NHE1 activity during ischemia and severe hypoxia. In the mammalian heart, NHE1 is activated by catecholamines, such as NE via α1-ARs (146, 321), possibly the α1A-subtype (see Ref. 14). Consistent with the postulate that α1-AR-mediated NHE1 activation is a central event in NE-mediated myocardial ischemic damage, NHE1 inhibition reversed the proarrhythmic effect of an α1-agonist in isolated rat hearts (340). With respect to the β-ARs, the picture is less clear, since both inhibition (146) and stimulation (67) of NHE1 in response to stimulation of β1-AR have been reported.

ANG II (244, 332) has been proposed to stimulate NHE1 in cardiac ischemia/reperfusion, although it is clear that ANG II is not the sole activator of NHE1 after myocardial infarct (257). ET-1 (which may, as noted above, be downstream from ANG II) has also been found to be upstream of NHE1 in the adverse effects of myocardial ischemia (35, 133).

ERK1/2 and/or the downstream effector p90RSK have been found to be involved in the stimulation of NHE1 by ischemia/
Reperfusion (182, 258). Supporting this notion, cardiac NHE1 stimulation by α1A-AR agonists, AT1, and thrombin were attenuated by blocking the ERK1/2 pathway (see Ref. 14). In rat hearts exposed to 30-min of no-flow ischemia and 30-min reperfusion, NHE1 was proposed to be directly phosphorylated by ERK1 following reperfusion, and by p90RSK during both ischemia and reperfusion (182). In contrast, p38 MAPK was found to negatively regulate NHE1 activity after ANG II stimulation in vascular smooth muscle (143).

Not only MAPKs, but also PKC, is implicated in stimulation of NHE1 by cardiac ischemia/hypoxia (243), as well as by a number of G protein-coupled, receptor-mediated signaling events characteristic of cardiac ischemia, including catecholamines (acting via α1-ARs), ANG II, ET-1, and thrombin (14).

Finally, several studies (194, 249, 258) have implicated ROS (specifically H2O2) as important mediators of NHE1 stimulation by cardiac ischemia/reperfusion, in some cases, via ROS-mediated ERK1/2 activation (249, 258).

A number of ischemia-associated signaling events have also been found to attenuate NHE1 activity. Estrogen stimulates the release of nitric oxide (NO), a mechanism suggested to underlie the protection of premenopausal women against myocardial ischemia (200). Recent evidence indicates that the protective effect of NO in cardiac ischemia, at least in part, involves inhibition of NHE1, an effect tentatively suggested to involve cGMP-dependent kinase (PKG) and p38 MAPK (8, 119). Histamine, acting via H1 receptors, which are inhibitory receptors in cardiac adrenergic nerve endings, was also found to inhibit NHE1, and this was proposed to be the mechanism of H1-mediated protection in myocardial ischemia (280). Interestingly, hypertonic pretreatment appears to attenuate NHE1 activity and exert protection during hypoxia/ischemia (71, 105). This is consistent with the notion that signaling is prioritized, such that when responding to a given stimulus (in this case cell volume), NHE1 will no longer respond to a second stimulus (in this case acidic pH; see section 2.4.1).

4.1.2.2 Consequences of stimulation of NHE1. Figure 2 illustrates some of the major consequences of NHE1 stimulation by cardiac ischemia/hypoxia. Elevated [Ca2+]i is generally considered the major cause of cell damage associated with myocardial ischemia (284). In 1985, Lazdunski and coworkers (149) proposed the “coupled exchanger” hypothesis, which stated that the ischemia-induced increase in [Ca2+]i results from influx, or decreased efflux, of Ca2+ via NCX as a consequence of the increase in [Na+], resulting from increased Na uptake via NHE. The Lazdunski model, based on cells in culture, has since been confirmed and extended by multiple workers in the field (10, 39, 117, 155, 190, 286; see Ref. 264).

Among these, the authors of this review have verified the model in both ischemia/reperfusion (7, 155, 156) and in hypoxia (10, 38, 39). In perfused hearts, NHE1 inhibitors attenuate the increases in [Na+]i and [Ca2+]i during ischemia, and improve [Na+]i and [Ca2+]i recovery on reperfusion (155, 286). Moreover, in rat hearts, an inverse correlation between Na+ accumulation during ischemia and functional recovery on reperfusion was demonstrated, and Na+ recovery on reperfusion was shown to be attenuated by low [Ca2+]i, or NCX inhibition (117). This led to the conclusion that NCX-mediated exchange of Ca2+ for Na+ during ischemia is a major mechanism of Ca2+ accumulation associated with reperfusion injury (117; see Fig. 2).

The role of pH i in the downstream events after stress-induced NHE1 activation is a complex issue. Recovery of pH i on reperfusion is attenuated by NHE1 inhibition in some (261, 286), although not all (155), studies of myocardial ischemia. However, paradoxically, several studies have shown that inhibition of NHE1 renders pH i unaltered or even less acidic in myocardial ischemia, presumably because the NHE1-dependent Na+ and Ca2+ accumulation stimulates ATP hydrolysis and thus H+ production (8, 155, 232, 261, 286; see also Ref. 264). Moreover, both beneficial and deleterious effects of acidic pH i during cardiac ischemia/reperfusion have been described. Elevation of pH i facilitates hypercontracture, and ischemia-induced cardiomyocyte damage and hypercontracture was inhibited both by blocking NHE1 and by inducing extracellular acidification, which, as noted above, can also inhibit NHE1 (26, 145). On the other hand, acidic pH i facilitates cell death by apoptosis, at least in part, because many apoptotic proteases and DNAses exhibit an acidic pH optimum (see also section 5.2).

Inhibition of NHE1 by 5′(N-ethyl-N-isopropyl)amiloride (EIPA) or cariporide has repeatedly been found to reduce ATP-depletion in cardiac ischemia (155, 232, 253, 261), and NHE1−/− mice exhibit better cardiac ATP preservation than wild-type mice, both at the end of ischemia and at the end of reperfusion (324). These findings strongly indicate that NHE1 activity contributes to ATP depletion in cardiac ischemia, presumably to a large extent, reflecting the energetic cost of, e.g., increased Na+–K+–ATPase and Ca2+–ATPase activity, resulting from the NHE1-dependent increases in [Na+]i and [Ca2+]i (for a discussion, see, e.g., Ref. 8).

In human neuroblastoma cells (a model for cardiac sympathetic nerve endings), ANG II acting on AT1 receptors was found to stimulate NHE1 activity, and this led to NE release by...
NET reversal from reuptake to release mode (244, 280). Thus it appears that excessive carrier-mediated NE release during myocardial ischemia/infarction may, at least in part, reflect reversal of the Na⁺-dependent NET in sympathetically innervated regions because of the NHE1-mediated increase in [Na⁺].

Interestingly, there is evidence that NHE1 can be upstream, rather than downstream, of MAPK activation in the heart also under conditions similar to those occurring in ischemia/reperfusion. Thus ANG II- and 5-HT-mediated ERK1/2 activation in rat aortic smooth muscle cells were mediated via NHE1-dependent Ras-Raf-MEK activation (187). In a rabbit model of nonischemic heart failure, p38 MAPK phosphorylation, as well as apoptosis, fibrosis, myocyte cross-sectional area, and intracellular nitric oxide synthase expression were all significantly reduced by treatment with the NHE1 inhibitor BIIB722 (3). In addition, NHE1 stimulation by cardiomyoctye stretch is upstream of stretch-induced Raf-1 and MAPK activation and subsequent hypertrophy (299, 333; see also above, section 3.1.1).

NHE1 inhibitors also attenuate cardiac myocyte swelling in ischemia (13). Moreover, NO and -OH release after cardiac ischemia/reperfusion in rat hearts was secondary to increases in NHE1 and NCX activity (164), suggesting that NHE1 stimulation in the heart may also be upstream rather than downstream of the ROS formation known to contribute to ischemia/reperfusion damage (section 4.1.1.2).

Finally, NHE1-mediated cardiac endothelial cell swelling could contribute to ischemic damage, e.g., by reducing capillary diameter, potentially diminishing RBC and leucocyte flow. Consistent with this postulate, in endothelial cells, NHE inhibitors attenuated swelling induced by lactacidsis (18) and by low-flow ischemia (171).

4.1.3 Mechanisms and Consequences of Stimulation of NKCC1

Furosemide and bumetanide inhibit Na⁺ influx and reduce injury during hypothermic ischemia in rat hearts (252), and findings in isolated perfused hearts from both rabbit and rat indicate that NKCC1 is stimulated by ischemia and remains active during reperfusion (7, 13, 252). Moreover, ischemic preconditioning stimulated NKCC1 in hearts of newborn rabbits (9). It is important to note that in marked contrast to the substantial driving force for Na⁺ influx via NHE1, the force driving NKCC1 can actually be directed out of the cell in cardiac ischemia as a consequence of the robust Na⁺ uptake via NHE1. In rabbit hearts perfused with ouabain-containing K⁺-free solution (i.e., outward directed driving force for NKCC1), bumetanide augmented [Ca²⁺], elevation during ischemia (1 h) and reperfusion, and inhibited [Na⁺], recovery on reperfusion. This indicates that under these conditions, NKCC1 contributes to Na⁺ efflux during reperfusion (7). Under normal [K⁺], conditions, data were consistent with the interpretation that in the early phase of ischemia, the driving force for NKCC1 is directed into the cell and NKCC1 contributes to ischemia-induced [Na⁺], elevation, whereas later in ischemia the driving force for NKCC1 is directed outward, and NKCC1 functions as a Na⁺ efflux pathway (7). Thus several lines of evidence point to activation of NKCC1 by cardiac hypoxia/ischemia. On the other hand, although direct comparisons of the relative roles of NHE1 and NKCC1 in myocardial ischemia/severe hypoxia are essentially lacking, there is evidence to suggest that at least in rat hearts, the role of NKCC1 may be more modest than that of NHE1 (52, 241).

4.1.3.1 Mechanisms of stimulation of NKCC1. The mechanism(s) of ischemia-induced NKCC1 stimulation in the heart have, to the knowledge of the authors, not been investigated directly. As noted above (section 2.4.2), NKCC1 is generally inhibited by acidic pH. This would tend to limit its activity under ischemic conditions, however, to our knowledge, the potential effect of pH, on NKCC1 activity during cardiac ischemia has not been studied directly.

Several signaling pathways known to be active in the ischemic heart (section 4.1.1.2) stimulate NKCC1 and thus may play a role in ischemic NKCC1 activation. In rat myocardium, NKCC1 was phosphorylated and activated by catecholamines via α₁-AR receptors in an ERK1/2 dependent manner (6). ANG II, aldosterone, and increases in [Ca²⁺], have also been proposed to stimulate the myocardial NKCC1 (see Ref. 6), and thrombin is a well-known activator of NKCC1 (215).

4.1.3.2 Consequences of stimulation of NKCC1. In diabetic rat hearts, in which basal NKCC1 activity is increased, NKCC1 was found to contribute to ischemic damage, whereas no contribution of NKCC1 to ischemic damage could be detected in nondiabetic rat hearts (241). Bumetanide inhibited the ischemia-induced increase in [Na⁺], in both control and diabetic hearts, but significantly more so in the latter, and only in diabetic hearts was NKCC1 inhibition associated with reduced ATP depletion and improved functional recovery on reperfusion (241). This suggests that, at least in the rat, the role of NKCC1 in cardiac ischemia may be modest; essentially no ouabain-insensitive ⁸⁶Rb⁺ influx was noted in another study of rat hearts exposed to low-flow ischemia (52). Whether this is particular to rats is, to our knowledge, unknown. Recent findings in newborn rabbit hearts suggest that NKCC1, when functionally coupled with Cl⁻/HCO₃⁻ exchange, can be linked to changes in pH during ischemia (that is, Cl⁻ entering on NKCC1 is recycled out of the cell via AE in exchange for HCO₃⁻ influx to elevate pH, or vice versa, if the driving force for NKCC1 is directed out of the cell) (9). Thus after preconditioning, acidification during ischemia was relatively decreased in the presence of bumetanide (9). Similarly, inhibition of NKCC1 was associated with increased pH during ischemia in the diabetic rat hearts (241). These results are consistent with the hypothesis that during late ischemia, after [Na⁺], and Cl⁻, have risen (7), the force driving NKCC1 is directed out of the cell, such that NKCC1 inhibition limits Cl⁻ and Na⁺ loss via NKCC1 and thereby functionally coupled HCO₃⁻ loss via AE.

4.2 Ischemia and Severe Hypoxia in the Brain

4.2.1 General Aspects

4.2.1.1 Energy status and ion homeostasis. Similar to cardiac ischemia, cerebral ischemia has been found to be associated with a marked decrease in cellular ATP levels (see Ref. 154). In cultured BBB endothelial cells on the other hand, ATP levels were unaltered through 4 h of hypoxia (the approximate time of edema formation in stroke), even at very low O₂ levels or in the absence of glucose, and after 24 h of hypoxia, ATP levels fell by only ~50% (81). Both pH₇ and pH in the brain of normoglycemic animals can fall by a full pH unit in hypoxia.
Intracellular space comprises about 80% of the total space occupied by the brain. In ischemia, major ionic shifts occur between the intra- and extracellular spaces, including, most notably, increased [Na\(^{+}\)], [Ca\(^{2+}\)], and [Cl\(^{-}\)], and decreased [K\(^{+}\)], accompanied by the corresponding inverse changes in these ions in the extracellular space (103, 154, 248). Similar to the situation in the heart, increases in [Na\(^{+}\)] during ischemia favor NCX-mediated Ca\(^{2+}\) influx and consequent Ca\(^{2+}\)-induced cell damage (111), and the rapid increases in [Na\(^{+}\)] and [Ca\(^{2+}\)], together with swelling of neurons, astrocytes, and endothelial cells are major causes of brain damage associated with limited perfusion (33, 90, 124, 135, 142, 177, 278, 288). The extent of [Na\(^{+}\)] elevation and cell swelling varies depending on location in the ischemic region, with the most pronounced effects occurring in the core of the ischemic zone and more moderate slower onset effects in the penumbral regions (130).

Under normal conditions, the BBB mediates Na\(^{+}\), Cl\(^{-}\), and water influx into the brain interstitial fluid and in so doing secretes (i.e., transports from blood into brain) up to 30% of the brain interstitial fluid (the remainder produced by the choroid plexus) (55). During the early hours of cerebral ischemia, brain edema forms by a process involving increased secretion of Na\(^{+}\) and water across an intact BBB (135, 178, 262). At the same time, ischemia stimulates NHE1- and/or NKCC1-dependent uptake of Na\(^{+}\), Cl\(^{-}\), and water into astrocytes, causing cytotoxic edema (31, 135, 144), a process likely facilitated by the increased BBB secretion of Na\(^{+}\), Cl\(^{-}\), and water into the brain interstitium (229). In other words, whereas BBB breakdown and consequent paracellular solute and water uptake does not occur until after prolonged ischemia (4–6 h), the Na\(^{+}\) and water uptake, which is the basis for brain edema in the early hours of ischemic stroke, occurs before BBB breakdown, indicating that it is transcellular and thus transporter mediated (22). As described above, both NHE1 and NKCC1 appear to play a role in the ionic shifts and cell damage elicited by cerebrovascular ischemia/hypoxia.

4.2.1.2 Signaling events. With the important exception of the elevated glutamate levels found in cerebral ischemia (154), many of the signaling events elicited by ischemia, anoxia, and prolonged hypoxia in the brain are similar to those described above for the heart. ANG II release resulting from activation of the brain renin-angiotensin system appears to be involved in brain damage elicited by ischemic stroke (56), and ET-1 release has also been found to contribute to ischemic cell damage in the brain (see Refs. 133 and 297). There is evidence for the involvement of centrally released arginine vasopressin (AVP) in brain ischemia and consequent cell damage (62, 275). Brain thrombin levels have also been proposed to be elevated after cerebral ischemia and to contribute to ischemic damage (113). Cytokines and inflammatory mediators released, at least in part, from astrocytes and brain microglia also play important roles, and specifically both IL-1 and IL-6 appear to contribute to ischemic brain damage (250, 343). As in the heart, changes in the activity of the MAPKs ERK1/2, JNK, and p38 MAPK (73), as well as of several other protein kinases, have been reported in the ischemic brain (see Ref. 154). Finally, ROS release in the brain is increased during both ischemia and (more so) during reperfusion, and this appears to contribute to ischemia/reperfusion-induced cell damage (see Ref. 154).

4.2.2 Mechanisms and Consequences of Stimulation of NHE1

Ischemic/anoxic/hypoxic NHE1 stimulation and protective effects of NHE1 inhibitors have been described in neurons from many brain regions, including mouse neocortical neurons (124), rat cortical neurons (317), and rat hippocampal CA1 neurons (270, 337), as well as in glial cells (27, 121, 234) and in brain endothelial cells (23, 109, 307). Moreover, protective effects of NHE1 inhibitors have been demonstrated in in vivo models of brain ischemia (317). Similar to what has been found in the heart, however, NHE1 is not the only culprit; NKCC1 (section 4.2.3), tetrodotoxin-sensitive Na\(^{+}\) channels (86), and Na\(^{+}\) influx via ionotrophic glutamate receptors (90) have been found to contribute to the ischemia-induced increase in [Na\(^{+}\)], in the CNS.

4.2.2.1 Mechanisms of stimulation of NHE1. In rat hippocampal CA1 neurons, NHE1 is stimulated by anoxia followed by reoxygenation, yet is apparently inhibited during the anoxia phase per se, possibly because of a reduced cellular ATP level under these conditions (270, 337). Similar to the situation in the heart, pH in the brain decreases very rapidly by up to a full pH unit in response to hypoxia (section 4.2.1.1); thus it appears likely that acidic pH contributes to stimulation of NHE1. On the other hand, in mouse astrocytes, in which OGD is associated with intracellular acidification (137), NHE1 activation during in vitro ischemia in mouse astrocytes was proposed to be independent of intracellular acidosis (136). Similarly, in these cells, the rate of pH\(_i\) recovery after intracellular acid loading by NH\(_4\) washout was increased by >80% after a 2-h exposure to OGD (137), suggesting that OGD-induced stimulation of NHE1 involves other mechanisms than an acidification-induced increase in activity.

Catecholamines, specifically NE, appear to play an important role in ischemia-induced increases in NHE activity in the brain via \(\beta\)- and \(\alpha\)-ARs and the cAMP/PKA pathway (282); however, at least in CA1 neurons, NE-mediated NHE activity was found to be amiloride insensitive (282). Hence, although NHE1 is also present in CA1 neurons and stimulated during anoxia (99, 337), the \(\beta\)-AR-stimulated NHE in these neurons may be NHE5, which is found almost exclusively in the CNS and which has been proposed to be the amiloride-insensitive brain NHE (240).

NHE1 activation during in vitro ischemia in mouse astrocytes has been proposed to be partially dependent on ERK1/2, although the upstream signal leading to ERK1/2 activation was not identified (136). Cytokines released during ischemia and severe hypoxia may also play a role in NHE activation in the brain, as IL-1 and IL-6 have been shown to stimulate NHE1 in astrocytes (21). In contrast to the proposed role of ROS in NHE1 activation during cardiac ischemia/reperfusion, the few available studies indicate that ROS may inhibit NHE activity in the brain (188, 309). Finally, in brain microvascular endothelial cells, NHE1 was reported to be stimulated by ET-1 (315).

4.2.2.2 Consequences of stimulation of NHE1. Elevation of [Ca\(^{2+}\)]\(_i\) is an important cause of the damage induced by ischemia and severe hypoxia in the brain (154, 277, 284). As in the heart, NCX reversal has been found to be involved in
ischemia-induced Ca\(^{2+}\) influx and consequent damage in both neurons and glial cells (27, 111, 150). The relative contributions of NHE1 and NKCC1 to the disruption of Ca\(^{2+}\) homeostasis in the ischemic brain have, to our knowledge, not been directly investigated. However, at least in mouse astrocytes, OGD-induced changes in Ca\(^{2+}\) homeostasis were mainly dependent on NKCC1 (150; see also section 4.2.3); hence, it appears that, whereas NHE1 is clearly a major player in disruption of Ca\(^{2+}\) homeostasis in the ischemic heart, NKCC1 may, in some cases, be more important in the brain. In glial cells, stimulation of NHE1 by ischemia/hypoxia or lactic acidosis and protective effects of NHE1 inhibition against glial cell swelling and death, under these conditions, have been demonstrated in a variety of preparations (27, 121, 234). Consistent with the view that NHE1 contributes importantly to the hypoxia-induced increase in Na\(^+\) transport across the BBB, NHE1 inhibition is protective against hypoxia-induced brain endothelial cell dysfunction and BBB disruption (23, 109, 307), and intravenous HOE-642 and bumetanide to inhibit BBB NHE1 and NKCC1 activity was recently found to decrease edema formation and cerebral infarct size in middle cerebral artery occlusion (MCAO) in the rat, apparently in an additive manner (307 and O‘Donnell ME, unpublished observation).

Stimulation of NHE1 during ischemia and severe hypoxia in the brain appears to ameliorate the decrease in pHi during both ischemia and reperfusion. In mouse neocortical neurons in primary culture, NHE1 inhibition exacerbated acidification during chemical anoxia and inhibited pHi recovery on “reperfusion” (124). Similar to the findings in the heart, a “pH paradox” of both protective and damaging effects of acidic pH has been described in the brain. Mild acidosis is protective in brain ischemia, at least partly, by attenuating Ca\(^{2+}\) influx via N-methyl-D-aspartate (NMDA) and \(\alpha\)-amino-3 hydroxy-5-methylisoxazole-4-propionic acid (AMPA) receptors and voltage-dependent Ca\(^{2+}\)-channels and by reducing energy demand and ATP depletion (102, 305), but likely also because of the inhibition of NHE1 by acid pHi (317). Acid pHi, which inhibits NHE1 activity, was neuroprotective in chemical anoxia in dissociated cortical cultures, apparently, at least in part, because of NHE1 inhibition (317). Consistent with the notion that NHE1 exhibits little activity during the ischemic phase per se, this effect of acidic pHi was only seen during reperfusion (317). Similarly, in mouse neocortical neurons, in which an EIPA-sensitive NHE is a major regulator of pHi after acidification, EIPA and acidic pHi both reduced pHi recovery (223), and acidic pHo (EIPA not tested) attenuated the increase in [Ca\(^{2+}\)]\(_i\) during chemical anoxia (124). Ischemia/reperfusion-induced free fatty acid release is shown to be reduced by inhibition of NHE1, perhaps because of a pH effect on phospholipase A\(_2\) (233). If, however, pHi is too acidic, this will favor cell death by apoptosis (section 5.2). Acidic pHi has also been demonstrated to potentiate ROS formation in the brain directly, to inhibit mitochondrial metabolism (thus accelerating ATP depletion), and to exacerbate neuronal death (338).

4.2.3 Mechanisms and Consequences of Stimulation of NKCC1

NKCC1 knockout mice exhibit reduced brain damage in stroke models (47), and, as described below, there is evidence for roles for NKCC1 in ischemic damage in neurons, astrocytes, and brain endothelial cells. Early studies provided evidence that a luminal Na\(^+\) transporter, working in tandem with abluminal Na\(^+\)/K\(^+\) pump and Cl\(^-\) efflux pathways, is rate limiting in BBB Na\(^+\) secretion (24, 262). More recent data strongly indicate that NKCC1 serves as such a luminal Na\(^+\) uptake pathway during ischemia. Thus NKCC1 resides predominantly in the luminal membrane of BBB endothelial cells in situ (205) and is stimulated by AVP (203), hypoxia (81), and aglycemia (81), prominent factors present during cerebral ischemia.

4.2.3.1 Mechanisms of stimulation of NKCC1. In the brain, mechanisms of NKCC1 activation in ischemia are better understood than in the heart, although far from fully elucidated. Elevated extracellular K\(^+\) and glutamate levels (154), both of which occur in cerebral ischemia (section 4.2.1) have been found to stimulate NKCC1 activity in neurons (Fig. 3A). Thus
activation of both ionotropic and metabotropic NMDA receptors, as well as of AMPA receptors, activated NKCC1 in cortical neurons, as did high \([K^+]_o\)-induced depolarization, both in a \(Ca^{2+}\)-dependent manner (17, 266). Elevated \([K^+]_o\) also increased NKCC1 activity in glial cells, similarly in a \(Ca^{2+}\)-dependent manner (290; Fig. 3B).

AVP was found to activate NKCC1 in brain endothelial cells in a \(Ca^{2+}\)-dependent manner associated with an increase in NKCC1 phosphorylation (see Ref. 203), and ET-1 activated NKCC1 in brain endothelial cells in a \(Ca^{2+}\)- and PKC-dependent manner (131; Fig. 3C). In this regard, it is interesting to note that NKCC1 was activated by ketocids, which can cause the release of ET-1 and/or increases in \([Ca^{2+}]_i\), in brain microvascular endothelial cells, and this mechanism appears to be important in the cerebral edema formation occurring in diabetic ketoadicosis (147).

Cytokines, long known as mediators of ischemic brain damage (section 4.2.1.2) may be involved in ischemia-induced increases in NKCC1 activity. Thus IL-6 released by astrocytes was shown to activate NKCC1 in brain endothelial cells (293), and the immunosuppressant FK506, which prevents IL-6 up-regulation in astrocytes and microgria, reduced infarct volume in a rat MCAO model (343).

Similar to other mechanisms of acute stimulation of NKCC1, ischemia-induced increases in NKCC1 activity are generally associated with increased NKCC1 phosphorylation on ser\(^{184}\) and ser\(^{189}\), as reported in rat cerebral cortex at 4–8 h of reperfusion after MCAO (334) and in mouse cortical astrocytes after 2 h of OGD (150). Similarly, both hypoxia and AVP increased the phosphorylation of NKCC1 in cultured bovine cerebral microvascular endothelial cells (81). The protein kinases that mediate ischemia-induced NKCC1 phosphorylation in the brain are unknown; however, several candidates are known to be activated in the ischemic brain (section 4.2.1.2).

Not only posttranslational regulation appears to be important, e.g., in rat model of focal cerebral ischemia/reperfusion (2-h MCAO and 24-h reperfusion), NKCC1 protein levels were significantly upregulated in the cortical neurons at the end of reperfusion (336), and similar findings were reported in whole rat cerebral cortex and striatum (334).

Interestingly, at least in BBB endothelial cells, a decrease in cellular ATP is not necessary for hypoxia-induced stimulation of NKCC1 (81). In these cells, hypoxia is a rapid and potent stimulator of NKCC1, counter to the common assumption that BBB cells are highly resistant to hypoxia (81). Simply removing glucose and pyruvate increases NKCC1 activity in the BBB cells under normoxic conditions (81; Fig. 3C). In the presence of pyruvate, glucose has no effect on the ability of hypoxia to stimulate NKCC1; however, if pyruvate is removed, NKCC1 activity is increased 2.6-fold both in normoxia and in hypoxia (81). Thus NKCC1 activity in BBB endothelial cells appears to be very sensitive to changes in metabolic profile, rather than just to a decrease in PO\(_2\) (81).

Finally, it may be noted that recent findings from O’Donnell, et al. (204) indicate that a reduction in NKCC1 activity in astrocytes and BBB endothelial cells, and consequent reduced edema formation, contributes to the neuroprotective effects of estradiol in stroke.

4.2.3.2 Consequences of stimulation of NKCC1. Studies of NKCC1 knockout mice (47), as well as studies employing intracerebral bumetanide administration, strongly indicate an important role of NKCC1 in ischemic brain damage. Bumetanide potently reduced neuron and astrocyte swelling and infarct volume after focal cerebral ischemia in rats (2-h MCAO followed by 24-h reperfusion) (336). Several studies have specifically addressed the involvement of NKCC1 in neuronal damage (Fig. 3A). NKCC1 inhibition was found to inhibit the increase in \([Na^+]_o\), cell swelling, and cell death elicited by OGD or glutamate in 14- to 15-day cortical neurons in vitro (17). In rat hippocampus, furosemide and bumetanide enhanced ATP recovery after OGD and attenuated CA1 neuronal injury (236). In astrocytes, NKCC1 is an important contributor to the cell swelling and excitatory amino acid (EAA) release in response to high \([K^+]_o\) (a condition prevalent in the ischemic brain, see section 4.2.1.1). In accordance with this, astrocytes from NKCC1\(^{-/-}\) mice exhibit absence of swelling and decreased EAA release after high \([K^+]_o\) (289, 290). The main mechanism in NKCC1-medicated EAA release appears to be that NKCC1-induced cell swelling activates the volume-expansion sensing outward rectifying anion channel (also known as the volume-regulated anion channel), through which EAA are released (255; Fig. 3B). In postnatal day 10 (P10), rat optic nerve astrocytes, OGD-induced cell swelling, and necrotic cell death were dependent on NKCC1 (302). In mouse cortical astrocytes, \(Na^+\) uptake by NKCC1 appeared to contribute to NCX-mediated \(Ca^{2+}\)-loading of intracellular \(Ca^{2+}\) stores during ischemia (Fig. 3B). Thus 2 h of OGD followed by 1 h of reperfusion elicited \([Na^+]_o\) elevation and cell swelling, both of which were attenuated by bumetanide and by genetic ablation of NKCC1 (150). OGD, moreover, elicited an increase in agonist-induced \(Ca^{2+}\) release, which was inhibited by bumetanide, absent in astrocytes from NKCC1\(^{-/-}\) mice, and inhibited by KB-R7943, an inhibitor of \(Ca^{2+}\) entry via NCX (150).

Increased NKCC1 activity in BBB endothelial cells also appears to contribute importantly to the detrimental effects of brain ischemia (Fig. 3C). In rat brain, NKCC1 was found predominantly on the luminal side of the microvascular endothelial cells, and administration of intravenous bumetanide to inhibit the BBB cotransporter significantly attenuates edema formation in the rat MCAO model of stroke, whether added 20 min before or after onset of MCAO (205, 306, 307). These findings underscore the important role for NKCC1 in mediating solute uptake across the BBB and suggest that ischemia-induced increases in NKCC1 activity elicits excessive solute uptake, resulting in brain edema. In contrast to findings in rat brains, where microdialysis of bumetanide into the cortices was only protective if administered presischemia (334), bumetanide has also been shown to reduce edema and infarct size in rat brain, even when administered after MCAO (306), i.e., the remaining 20% perfusion during MCAO is sufficient to deliver bumetanide to the BBB NKCC1. This is of obvious clinical interest, as it indicates that bumetanide given after the onset of stroke may still be beneficial in reducing edema and infarct size.

As noted above (section 3.2.2), \([Cl^-]\) is a major determinant of GABAergic signaling (increased \([Cl^-]\), being associated with attenuation or even reversal of the inhibitory effect of GABA, and thus increased excitability). It is thus likely that the detrimental effect of increased NKCC1 activity in the ischemic brain may, at least in part, reflect elevation of \([Cl^-]\); however, this has, to our knowledge, not been directly investigated.
Finally, another consequence of increased NKCC1 activity may be modulation of the activity of stress-activated protein kinases. NKCC1 associates directly with SPAK and the related kinase oxidative stress response 1 (230, 231), members of the germline kinase subfamily of the ste 20-related kinases, both of which are expressed in the brain (230). In brain slices, NKCC1, SPAK, and p38 MAPK were found to form a complex from which p38 MAPK appeared to dissociate on ischemic stress, leading to the proposal that NKCC1 may play a scaffolding role in regulating the cellular stress response (230).

4.3 Ischemia and Severe Hypoxia in the Blood

4.3.1 General Aspects

In their capacity as \( \text{O}_2-\text{CO}_2 \) transporters, RBCs play a central role in the responses to hypoxic stress. Although this review only deals with the possible role of RBCs, it should also be noted that both platelets (180) and leukocytes contribute importantly to the response to ischemia/hypoxia (see Refs. 139 and 154).

4.3.1.1 Energy status and ion homeostasis. In mouse RBCs, cellular ATP levels were unaffected by 1 h of hypoxia (0.5% \( \text{O}_2 \)) (25). In RBCs with mitochondria (e.g., nucleated RBCs from birds or teleosts or mammalian reticulocytes), low oxygen conditions can elicit a shift from oxidative to glycolytic metabolism and are associated with decreased ATP levels (although this may, in fact, not be caused by decreased oxidative metabolism, see e.g., Ref. 198). Even in nonnucleated RBCs, glycolysis is increased by deoxygenation (179; see Ref. 89).

In contrast to the pHi decrease induced by deoxygenation in the heart and brain (see sections 4.1.1.1 and 4.2.1.1), deoxygenation per se will lead to an increased pHi of RBCs because of the Haldane effect (the effect of oxygenation on the \( \text{H}^+ \)-binding properties of Hb) (197). Deoxygenation, moreover, increases free cytoplasmic \( \text{Mg}^{2+} \) concentration purely as a result of the Hb oxy-deoxy changes (79, 239). Finally, some cell swelling will occur in hypoxic RBCs solely because of the Cl\(^-\) shift induced by deoxygenation (75). If NKCC1 and/or NHE1 is stimulated under conditions in which the driving force favors \( \text{Na}^+ \) influx via these transporters, deoxygenation is expected to increase [\( \text{Na}^+ \)], and [\( \text{Cl}^- \)], and in some cases also [\( \text{K}^+ \)], and elicit further cell swelling. Precisely such effects were reported in RBCs from several species, including trout and turkey (75, 192). On the other hand, [\( \text{Na}^+ \)], and [\( \text{K}^+ \)], in mouse RBCs were reported to be essentially unaffected by 45 min of hypoxia, as well as by 15-min hypoxia followed by 15-min reoxygenation (25). With respect to [\( \text{Ca}^{2+} \)], this was reported to be elevated in RBCs for several weeks after myocardial ischemia in human patients (118). Oxidative stress or energy depletion of mammalian RBCs also appears to be associated with increased [\( \text{Ca}^{2+} \)] (148). On the other hand, experimental deoxygenation of human RBCs had no detectable effect on [\( \text{Ca}^{2+} \)] (303).

4.3.1.2 Signaling events. RBCs are obviously exposed to the many extracellular mediators released to the bloodstream during ischemia and severe hypoxia, including, e.g., catecholamines (adrenaline, noradrenaline), ET-1, AVP, dopamine, and atrial natriuretic peptide (ANP), as measured in human patients following cerebral infarction or stroke (16, 70, 193). The extent to which the RBCs have functional receptors for these mediators is likely to differ with species and RBC age/developmental stage. For instance, it has been reported that mature human RBCs lack functional receptor-coupled adenylate cyclase systems (72), although, in apparent contrast to this, NHE1 in human RBCs was reported to be stimulated by catecholamines, in part, via \( \beta_2 \)-receptors (30).

Numerous studies point to important roles of protein kinases in the oxygen-dependent regulation of NHE1 and NKCC1 in RBCs (section 3.3); however, the molecular identity of these putative hypoxia-activated kinase(s) is essentially unknown. Changes in redox systems may also be important signaling events in deoxygenated RBCs. In RBCs, ROS are formed to a degree proportional to \( \text{PO}_2 \), because of the high levels of ferrous iron and reducing enzymes (50). Therefore, hypoxia should decrease ROS production and prevent oxidation of reduced glutathione (GSH). Consistent with this, an increase in GSH levels was noted in mouse RBCs exposed to hypoxia (25).

4.3.2 Mechanisms and Consequences of Stimulation of NHE1

As pointed out above (section 3.3.1), it is well established that NHE1 is activated by hypoxic conditions in RBCs from lower vertebrates (89). It seems highly probable that this is the case also in mammalian RBCs, since multiple factors known to stimulate NHE1 are released to the bloodstream during ischemia in mammals (section 4.3.1.2). However, studies directly addressing the effect of hypoxia on NHE1 in mammalian RBCs appear to be lacking. In mammals, ischemia-induced NHE1 activation may only be of real consequence in RBCs, such as those of rabbits and dogs, or in reticulocytes, which exhibit robust transporter activities, since NHE1 activity in mature human RBCs is relatively minor (41, 123, 216; see also Ref. 222).

4.3.2.1 Mechanisms of stimulation of NHE1. In the RBCs of lower vertebrates, NHE1 is activated by deoxygenation per se, by a mechanism that has yet to be fully understood but may involve changes in redox systems, membrane-bound Hb, and protein kinase activity (section 3.2.3, and for an in-depth discussion, see Ref. 89).

In addition to this, hypoxic stimulation of NHE1 in the RBCs of most teleosts is also dependent on circulating catecholamines (see section 3.3.1). In flounder RBCs, catecholaminergic stimulation of NHE1 was, at least in part, PKA-dependent, and was mediated by a pathway distinct from that involved in stimulation of NHE1 by osmotic cell shrinkage or by inhibition of PP1 and PP2A (108). The elevated plasma level of catecholamines that results from ischemic stress in mammals (section 4.3.1.2) has also been reported to increase NHE1 activity in human RBCs, possibly, via both \( \alpha_1 \)- and \( \beta_2 \)-ARs (30), although this may be of minor consequence given the low NHE1 activity in these cells. Similarly, ANP, the plasma level of which is increased in ischemia (section 4.3.1.2) has been reported to stimulate NHE1 in human RBCs, an effect proposed to be mediated via cGMP (228).  

\(^2\) It may be noted that SPAK is a regulator of NKCC1 (63, 87), and its role in ischemia-induced NKCC1 activation is yet unclear.
In contrast to the prominent role of intracellular acidification in ischemic/hypoxic NHE1 stimulation in the heart and brain, acid pH, is unlikely to be a major pathway for NHE1 stimulation in ischemic/hypoxic RBCs. Although plasma pH, can be decreased in extreme hypoxia (75), deoxygenation per se will tend to increase pH, in RBCs because of the Haldane effect (section 4.3.1.1). Moreover, Hbs of mammalian RBCs exhibit very high H⁺-buffering capacities; thus, the effect of anoxia on pH, is likely to be limited (40). ROS may also be unlikely to mediate hypoxic/ischemic stimulation of NHE1 in RBCs, since H₂O₂ was found to inhibit NHE1 in trout RBCs (199).

4.3.2 Consequences of stimulation of NHE1. In teleosts, hypoxic NHE1 activation in RBCs will elicit an elevation of pH, and cell swelling, both of which will increase the O₂ affinity and saturation of Hb by virtue of the Bohr and Root effects (197). In humans, these effects could, in principle, apply but are likely to be modest because of the limited net NHE1 activity in mature human RBCs and the different Hb properties in mammals compared with teleosts (see above, and for a discussion, see Ref. 222). Reduced RBC deformability reduces blood flow (217), an effect that has been proposed to contribute to ischemic damage (304). The RBC swelling resulting from hypoxic activation of NHE1 could therefore in principle elicit detrimental effects on blood rheological properties, although this has yet, to our knowledge, to be directly investigated. It may also be noted that RBC lysis, which could be caused or facilitated by severe RBC swelling, contributes to ischemic damage, e.g., as shown in rat brain (113).

4.3.3.1 Mechanisms of stimulation of NKCC1. In RBCs from birds, ferrets, and humans, NKCC1 is stimulated by deoxygenation per se (64, 79, 192; see Ref. 89). On the other hand, in mouse RBCs, NKCC1 was reported to be unaffected by hypoxia (25), pointing to the existence of species-specific effects of hypoxia on NKCC1 activity.

Similar to that of NHE1, NKCC1 activity is modest in mature mammalian RBCs compared with the activity in reticulocytes or in nucleated RBCs (64, 166). On the other hand, at least under conditions of robust driving forces and high hematocrit, NKCC1 activity is high enough even in mammalian RBCs to elicit significant physiological effects (see Ref. 89), although little direct evidence is available for ischemia and severe hypoxia, as discussed below (section 4.3.3.3).

4.3.3.2 Consequences of stimulation of NKCC1. As described in section 3.3.3, hypoxia-induced NKCC1 activation in RBCs will, under conditions of an inwardly directed driving force for this transporter, tend to elevate K⁺ and may serve to buffer increases in plasma [K⁺]. Similarly, RBC swelling resulting from increased NKCC1 activity will increase the O₂ affinity and saturation of Hb but could also cause rheological problems, as noted above for NHE1 (section 4.3.2.2). Also, as discussed above, the gradient for NKCC1 is not always inwardly directed in RBCs (see sections 3.3.2 and 4.3.3.2 and also Ref. 166), such that in ischemia and severe hypoxia, where shifts in intra- and extracellular [Na⁺] and [K⁺] occur, the direction of transport via NKCC1 is hard to predict. Under conditions eliciting substantial outward flux via NKCC1, this could, in principle, contribute to apoptotic RBC shrinkage and ultimately death (section 5); however, to our knowledge, the relevance of this phenomenon to ischemia and severe hypoxia has not been directly studied.

5 NHE1- and NKCC1-Mediated Cell Death in Ischemia and Severe Hypoxia

5.1 Cell death in ischemia and severe hypoxia—apoptosis or necrosis? Apoptosis and necrosis are classically described as two distinct forms of cell death with fundamentally different characteristics. Cell death by apoptosis involves membrane blebbing, outer membrane leaflet inversion, nuclear condensation, and cell shrinkage (74). In contrast, necrosis is characterized by cell swelling and loss of plasma membrane integrity, resulting in the release of cell content to the surrounding milieu (74). As described above, cell swelling is a characteristic feature of ischemia/reperfusion damage in both the heart and the brain (see Refs. 120, 327, and 345), and swelling of RBCs on deoxygenation stress has also been reported (75). In part, for this reason, cell death as a consequence of ischemia was initially thought to be largely necrosis; however, accumulating evidence indicates that both apoptosis (or apoptosis-like, caspase-independent cell death) and necrosis contribute to ischemic cell death in both the heart (211, 345) and brain (93, 196). Whether cells exposed to ischemia or severe hypoxia die by apoptosis or necrosis, by a combination of both, or by some pathway unique to this condition, is a point of some controversy, and is probably dependent on multiple factors, such as cell type and severity of the ischemic insult (see, e.g., Refs. 120 and 196). For instance, apoptosis is an ATP-dependent process, and if ATP levels fall below a critical level, certain stimuli normally leading to apoptotic cell death will instead cause death by a necrotic pathway (see Ref. 74). The increase in [Ca²⁺], resulting from ischemia and severe hypoxia will activate death effectors, including Ca²⁺-dependent proteases and initiate mitochondrial apoptotic death pathways. Similarly, ROS are important mediators of apoptosis, in part, because of mitochondrial damage (157). Studies in cardiomyocytes indicate that the combination of hypoxia and low pH, prevalent in the ischemic heart synergistically trigger a unique death path-
way involving the Bcl-2 family death-promoting protein BNIP3 (94). Mammalian RBCs, which lack nuclei and mitochondria, do not undergo classical caspase-dependent apoptosis, yet die following energy depletion or oxidative stress by a caspase-independent form of cell death exhibiting many characteristics of apoptosis, and due, at least in part, to \( \text{Ca}^{2+} \) influx and increased \( [\text{Ca}^{2+}]_i \) (148).

5.2 Roles of NHE1. Consistent with a role for NHE1 in ischemia-induced cell swelling in both the heart and the brain, amiloride inhibited cold ischemia-induced cell swelling in rat hearts (13), and genetic ablation of NHE1 inhibited OGD-induced astrocyte swelling (137). NHE1 inhibitors have also been shown to attenuate apoptosis in a number of models of myocardial hypoxia/ischemia and heart failure (3, 15, 44, 294). In one study, cariporide was reported to inhibit ischemia/reperfusion-induced cardiomyocyte death by necrosis, as well as apoptosis (211). The NHE1-mediated necrosis is presumably because of \( \text{Na}^+ \) influx and concomitant cell swelling (Fig. 2). Only a few studies have addressed the mechanisms by which increased NHE1 activity leads to apoptotic cell death in ischemia. In neonatal rat cardiomyocytes, the NHE1-mediated increase in \( [\text{Ca}^{2+}]_i \) was proposed to lead to mitochondrial \( \text{Ca}^{2+} \) overload and activation of a mitochondrial death pathway (294, 300). NHE1-mediated mitochondrial \( \text{Ca}^{2+} \) overload after in vitro ischemia was also recently reported in mouse astrocytes (136). In cerebellar granule neurons, inhibition of NHE1 alone was found to elicit caspase-independent cell death with morphological features most characteristic of apoptosis (parapoptosis) (263). The role of NHE1 in apoptosis is thus not straightforward, and, additionally, antiapoptotic effects of NHE1 have been described in numerous studies of nonischemia conditions (246, 301, 328). An important protective effect of NHE1 in such cases appears to be maintaining \( \text{pH}_i \) above the acidic \( \text{pH}_i \) optimum for many death effectors, including caspases and cathepsins (169, 251, 263). Although this is in apparent contrast to the consistently protective effect of NHE1 inhibition in ischemia, it should be kept in mind that inhibition of NHE1 may, in fact, somewhat counterintuitively lead to increased \( \text{pH}_i \) (section 4.1.2.2).

5.3 Roles of NKCC1. Very few studies to date have addressed the potential involvement of NKCC1 in apoptotic vs. necrotic death following ischemic/hypoxic insults. An important consequence of NKCC1 stimulation by ischemia and severe hypoxia in the brain is neuronal and glial swelling and edema (section 4.2.3.2), pointing to a possible role of NKCC1 in ischemia/hypoxia-induced brain cell necrosis (Fig. 3). To our knowledge, only one study has specifically addressed this issue. The findings indicated that NKCC1 inhibition reduced necrosis, but not apoptosis, after focal ischemia in rat cerebral cortex (334). A few nons ischemia studies in tissues other than heart, brain, or blood have implicated NKCC1 in apoptotic cell death (134, 168). In RBCs, outward transport via NKCC1 and concomitant isotonic cell shrinkage has been tentatively proposed to play a role in the apoptotic process (158). A major cause of cell damage and death. The overall aim of this review was to evaluate and compare the roles of NHE1 and NKCC1 in the detrimental events resulting from ischemia and severe hypoxia. Specifically, we addressed the similar roles of these transporters in \( [\text{Na}^+]_o \) overload leading to NCX reversal, increased \( [\text{Ca}^{2+}]_i \), and consequent cell death via \( \text{Ca}^{2+} \)-dependent mechanisms, including mitochondrial death pathways. As discussed above, this scheme contributes importantly to the detrimental roles of both of these transporters in ischemia and severe hypoxia in the heart and brain, and there is also evidence to suggest a role in the blood. However, in all of the three tissues, there is increasing evidence for a major involvement of a number of other mechanisms in NHE1- and NKCC1-mediated damage, including changes in \( \text{pH}_i \) and cell volume, activation of MAPKs, and release of EAAs and ROS. Moreover, it appears that a complex interplay of multiple ischemia/hypoxia-activated signaling events mediates the activation of NHE1 and NKCC1 under these conditions, although the activation pathways are still incompletely described. The mechanisms leading to NHE1 and NKCC1 activation and the mechanisms by which activation of these transporters lead to cell damage and death in ischemia and severe hypoxia constitute potentially interesting targets for therapeutic intervention and should be a focus for future research in this clinically very important field.

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