[Frontiers in Bioscience 12, 762-770, January 1, 2007]

The role of membrane ion transport proteins in cerebral ischemic damage

Douglas B. Kintner[1], Yanping Wang[1], and Dandan Sun [1, 2]

[1]Depts. of Neurosurgery and [2]Physiology, Univ. of Wisconsin School of Medicine and Public Health, Madison, WI 53792

TABLE OF CONTENTS

1. ABSTRACT

Loss of ion homeostasis plays a central role in pathogenesis of ischemic cell damage. Ischemia-induced perturbation of ion homeostasis leads to intracellular accumulation of Ca^{2+} and Na^+ and subsequent activation of proteases, phospholipases, and formation of oxygen and nitrogen free radicals. This signal transduction cascade results in long-term functional and structural changes in membrane and cytoskeletal integrity and eventual cell death (1). Both ion conductances and ion transporters could affect ion homeostasis. Considerable research effort has been centered on roles of passive fluxes via cation and anion conductances in cerebral ischemic damage. This review will instead focus on the recent studies into the role of secondary active transport proteins in ischemia-induced dissipation of ion homeostasis. Secondary active ion transport proteins are a membrane protein-mediated solute transport mechanism that derives its energy from the combined chemical gradients of the transported ions. They are important in maintaining steady-state intracellular ion concentrations. These include Na^+-dependent chloride transport (NKCC), Na^+/H^+ exchange (NHE), and Na^+/Ca^{2+} exchange (NCX). Results from both *in vitro* and *in vivo* experimental studies suggest that these ion transport proteins are potential targets to reduce or prevent ischemia-mediated loss of ion homeostasis.

2. INTRODUCTION

Loss of ion homeostasis plays a central role in pathogenesis of ischemic cell damage. Ischemic-induced perturbation of ion homeostasis leads to intracellular accumulation of Ca^{2+} and Na^+ and subsequent activation of proteases, phospholipases, and formation of oxygen and nitrogen free radicals. This cascade of signal transduction events result in long-term functional and structural changes in membrane and cytoskeletal integrity and eventual cell death (1,2). Normally, the physiologically important ions (Na^+, Ca^{2+}, Cl^-) are not in electrochemical equilibrium across the plasma membrane. Their gradients are maintained by two major primary active transport proteins Na^+/K^+-ATPase and Ca^{2+}-ATPase. Primary active transport systems expends metabolic energy in ATP to transport specific ions against their concentration or electrochemical gradients. For each ATP molecule hydrolyzed, Na^+/K^+-ATPase usually drives 3 Na^+ ions out of cells and two K^+ ions in. The Ca^{2+}-ATPase provides the primary mechanism for removing Ca^{2+} from the cell. The activity of these pumps results in an extracellular fluid high in Na^+ (~ 145 mM) , Cl^- (~ 100 mM) and Ca^{2+} (~2 mM), but low in K^+ [~ 4.5 mM, (3)]. In contrast, the intracellular fluid is low in Na^+ (~10-15 mM), Cl^- (6-40 mM), and Ca^{2+} (< 10^{-7} M), but high in K^+ (~ 150 mM).

Secondary active ion transport proteins do not use energy stored in ATP directly. They are a membrane protein-mediated solute transport mechanism that derives its energy from the combined chemical gradients of the transported ions which are generated by Na^+/K^+-ATPase and Ca^{2+}-ATPase. Secondary active ion transport proteins are important in maintaining steady-state intracellular ion concentrations. These include Na^+-dependent chloride transport (NKCC), Na^+/H^+ exchange (NHE), and Na^+/Ca^{2+} exchange (NCX). During ischemia, cells lose K^+ and gain Ca^{2+}, Na^+, Cl^-, along with H_2O (1). This may result from a halt in ATP synthesis and extensive pump failure after anoxia. Both ion conductances and ion transporters could affect ion homeostasis during ischemia. Considerable research effort has been centered on roles of passive fluxes via cation and anion conductances in dissipation of the ion concentration gradients. Recently, Xiong et. al. have demonstrated a role for a glutamate receptor-independent, Ca^{2+}-permeable acid-sensing ion channel (ASIC) in neuronal injury (74). This review will instead focus on the recent studies on the role of secondary active transport proteins in ischemia-induced dissipation of ion homeostasis. Results from both *in vitro* and *in vivo* experimental studies suggest that these ion transport proteins are potential targets to reduce or prevent ischemia-mediated loss of ion homeostasis.

3. Na^+-DEPENDENT CHLORIDE TRANSPORTER

The electroneutral NKCC protein transports Na^+, K^+ and Cl^- into cells under physiological conditions with a stoichiometry of $1Na^+$: $1K^+$: $2Cl^-$ (4). The NKCC is an example of the secondary active transport where the transmembrane chemical gradients for Na^+, K^+, and Cl^- determine net inward ion movement in the cell (4). NKCC is characteristically inhibited by the sulfamoylbenzoic acid "loop" diuretics, such as bumetanide and furosemide. To date, only two distinct isoforms, NKCC1 and NKCC2, have been identified. NKCC1 has a broad tissue distribution, while the NKCC2 isoform is only found in vertebrate kidney.

3.1. NKCC1 plays a role in *in vitro* ischemic neuronal damage

The NKCC1 protein is expressed in neurons throughout the brain (5). It is well established that glutamate-mediated acute excitotoxic neurodegeneration is dependent on Na^+ and Cl^- entry (6, 7). We found that in cortical neurons activation of NMDA receptors triggers a significant increase in $[Na^+]_i$ and intracellular ^{36}Cl content accumulation. We also showed that activation of NMDA and AMPA receptors stimulates NKCC1 activity in neurons (8). Blocking of NKCC1 activity with bumetanide abolishes the Cl^- accumulation and attenuates the $[Na^+]_i$ rise by 52% (9). Bumetanide also inhibits both glutamate and NMDA-mediated cell death in cortical neurons (9). Thus, NKCC1 may act as one of the mechanisms that contribute to overload of Na^+ and Cl^- during glutamate-mediated acute excitotoxicity (9).

It is established that oxygen-glucose deprivation (OGD)-induced neuronal death in mature neurons is mediated by NMDA receptor-triggered excitotoxicity (10). After 3 h OGD and 21 h reoxygenation (REOX), 70% of cultured cortical neurons die. OGD-mediated neuronal cell death is significantly attenuated by bumetanide, confirming the role of NKCC1 in excitotoxicity (9). Similar protection is found in cortical neurons from NKCC1 null mouse ($NKCC1^{-/-}$) following OGD (11). No additional neuroprotection is found when 10 μM bumetanide is administrated in $NKCC1^{-/-}$ neuron cultures (11). These results imply that stimulation of NKCC1 activity is important in ischemic neuronal damage.

3.2. NKCC1 contributes to cell swelling, swelling-mediated EAA release and cell damage of cultured astrocytes

Expression of NKCC1 protein is found in astrocytes and oligodendrocytes (5,12,13). During ischemia, one significant pathophysiological change in the central nervous system (CNS) is an elevation of extraacellular K^+ ($[K^+]_o$). A few minutes of anoxia/ischemia raises $[K^+]_o$ to ~60 mM (1). NKCC1 in astrocytes may play a role in K^+ uptake under high $[K^+]_o$ conditions. In cultured astrocytes, 75 mM $[K^+]_o$ causes NKCC1-mediated K^+ influx to be stimulated by ~ 80% (14). 75 mM $[K^+]_o$ also triggers cell swelling in $NKCC1^{+/+}$ astrocyte by 20% (14). This high $[K^+]_o$-mediated swelling is abolished by inhibition of NKCC1 activity (14,15). Thus, our studies suggest that NKCC1 activation leads to high $[K^+]_o$-induced astrocyte swelling, glutamate release as well as Na^+, and Cl^- influx (14,15).

In addition, high $[K^+]_o$-induced astrocyte swelling is also observed in the rat optic nerve model (16). In enucleated nerves, astrocyte swelling is monitored by progressive increases in light transmittance in response to high $[K^+]_o$. Furosemide and bumetanide reversibly suppress the high $[K^+]_o$-induced astrocyte swelling (16).

Recently, we reported that NKCC1 and the reverse-mode operation of NCX contribute to intracellular Na^+ and Ca^{2+} overload in astrocytes following OGD/REOX (17). Loss of intracellular Na^+ homeostasis in astrocytes primarily results from excessive Na^+ entry via NKCC1 and NHE1 during REOX. Moreover, increases in intracellular Na^+ may evoke reversal of NCX and subsequent Ca^{2+} dysregulation (17,18). We further revealed that astrocyte death involves Na^+ and Ca^{2+} overload, dissipation of the mitochondrial membrane potential, and release of mitochondrial cytochrome C (18). These findings suggest that the concerted activities of multiple ion transport proteins are important in the perturbations of Na^+ and Ca^{2+} homeostasis and in astrocyte death that occurs in response to hypoxia/ischemia.

3.3. NKCC1 is involved in focal cerebral ischemia

We reported that inhibition of NKCC1 activity significantly reduces infarct volume and cerebral edema following cerebral focal ischemia induced by middle cerebral artery occlusion [MCAO, (19)]. Total NKCC1 protein expression is increased in cortex after 2 h ischemia followed by 0 , 4, 8, 12 and 24 h reperfusion of spontaneously hypertensive rats (SHR). In striatum, the significant increase is found during 4, 8, 12 and 24 h

reperfusion but not at the end of 2 h ischemia. Phosphorylated NKCC1 is also increased in cortex at 4 h reperfusion and peaks at 8 h reperfusion. Because changes in phosphorylation state of NKCC1 are a major regulatory mechanism that stimulates NKCC1 activity, these findings suggest that NKCC1 may be involved in cerebral ischemic damage. When 100 µM bumetanide is continuously microdialyzed into the left cortices throughout ischemia-reperfusion injury, infarct area is significantly reduced and the water content increase is decreased by 70% (19). This indicates that NKCC1 contributes to cerebral ischemic cell damage and brain edema. However, no protection is found if bumetanide is administered only during reoxygenation (19)(Yan et al, 2003), implying that activation of NKCC1 may play a role in the early stage of ischemic damage. Administration of bumetanide (7.6-30.4 mg/kg) intravenously immediately before occlusion attenuates the decrease in apparent diffusion coefficient ratios for both cortex and striatum (by 40-67%), reflecting a reduced edema formation in rats subjected to permanent MCAO model (20). This further suggests a role for NKCC1 in the edema formation during cerebral ischemia.

In addition to pharmacological blockage of NKCC1, we also used genetic ablation of NKCC1 to further establish that NKCC1 contributes to ischemic damage. In this study, infarct volume in $NKCC1^{-/-}$ mice is reduced by ~30 to 46% ($p < 0.05$) at 10 h and 24 h reperfusion after 2 h MCAO (11). NKCC1 is expressed in neuronal axons, astrocytes, as well as oligodendrocytes. We further investigated the role of NKCC1 in white matter damage. Amyloid precursor protein (APP) has been suggested as a sensitive marker for axonal transport disruption in white matter during brain ischemia (21). An increase in APP occurs in $NKCC1^{+/+}$ mice after 2 h ischemia and 10 h reperfusion, but less APP accumulation is found in $NKCC1^{-/-}$ mice [$p < 0.05$, (11)]. This implies that NKCC1 is involved in both gray and white matter damage after focal cerebral ischemia.

4. Na^+/H^+ EXCHANGER

NHEs catalyze the electroneutral exchange of H^+ and Na^+ ions (1:1 stoichiometry) across cellular membranes and down their concentration gradients, thereby regulating the pH of the cytoplasm or organellar lumen (22,23). To date, nine NHE family members have been identified in mammals. NHE1-5 are expressed on the plasma membrane in various cell types. NHE6-9 reside on intracellular organellar membranes of the endosomal/trans Golgi network (22,23). NHE1 is an ubiquitously expressed plasma membrane protein and the most abundant NHE isoform in the CNS (24). NHE1 serves the crucial function of protecting cells from internal acidification. Two major classes of pharmacological agents are currently used to inhibit NHE activity. One class includes amiloride and its 5' alkyl-substituted derivatives such as ethylisopropylamiloride (EIPA) or dimethylamiloride. Another dass of inhibitors includes the derivatives of the benzoylguanidines such as HOE 694 and HOE 642. Of these, HOE 642 is the most potent one, being 10^5 times more specific for NHE1 vs. NHE3 (25).

4.1. NHE is important in neuronal damage during *in vitro* ischemia

A number of investigations have concluded that NHE activity in neurons is enhanced in response to ischemic/hypoxic conditions as well as during subsequent REOX. In acutely isolated CA1 neurons from NHE1 wild-type ($NHE1^{+/+}$) mice, 5 min chemical anoxia (induced by sodium dithionite) triggers a large alkalization that could be blocked either by the NHE1 antagonist HOE 694 or by removal of external Na^+ (26). The anoxia-induced alkalization is also significantly reduced in $NHE1^{-/-}$ CA1 neurons. The protein kinase inhibitors chelerythrine, H-7, and H-89 significantly attenuate the increase in pH_i during anoxia, suggesting that the increase in NHE1 activity involves enhanced PKA and PKC activity (26). In a similar manner, Sheldon and Church (27) observe that acutely isolated CA1 neurons exhibit a tri-phasic response to 5 min anoxia: an initial acidification during anoxia, an alkalization as anoxia continues, and then a further alkalization upon REOX. Removal of external Na^+ or reducing the external pH attenuates the post-anoxia alkalization (27). The PKA inhibitor Rp-cAMPS can block the post-anoxia alkalization in CA1 neurons which suggests involvement of cAMP- dependent signaling pathways in NHE activation. When ATP levels fall to 35% of control in isolated CA1 neurons during chemical anoxia, NHE activity is reduced by 44%. Thus, activation of NHE function appears to be dependent upon ATP energy levels which support phosphorylation of the protein (27).

When cortical neuronal cultures are subjected to the metabolic inhibitors 2-DG and KCN for 20 min, pH_i decreases by 0.2 pH units, but then recovers when inhibition is removed (28). The post-inhibition pH_i recovery is significantly reduced by the NHE1 inhibitors dimethylamiloride or harmaline. The authors concluded that there is significant increase in NHE1 activity both during and following metabolic inhibition. Metabolic inhibition in cultured cortical neurons induced by sodium azide causes an initial decrease in pH_i within 2 min followed by a variable response, with roughly half the neurons alkalizing above baseline and half failing to regulate pH_i (29). When NHE is blocked, either with pH_o 6.6 or with EIPA, the decrease in pH_i during metabolic inhibition is three times larger and none of the neurons exhibit regulation of pH_i. These studies suggest that NHE1 activity is stimulated during and following metabolic inhibition.

In a recent study, we found that NHE1 is essential in pH_i regulation in cultured cortical neurons in response to an internal acid load (30). In addition to intracellular proton content, activation of NHE1 activity also affects cellular Na^+ levels. 2 h OGD results in a small (~ 2 fold) but significant increase in neuronal $[Na^+]_i$, which reaches to ~ 7 fold increase during 1 h REOX. The rise in $[Na^+]_i$ following OGD is significantly attenuated by HOE 642. $NHE1^{-/-}$ neurons do not exhibit significant increase in $[Na^+]_i$. This finding is consistent with the reports that NHE1 activity is elevated upon REOX (27, 29). 3 h OGD and 21 REOX lead to cell death in ~ 70% of the $NHE1^{+/+}$ neurons. However, cell death is significantly reduced in

wild type neurons treated with HOE 642 or in $NHE1^{-/-}$ neurons (30). Taken together, these studies suggest that NHE1 activity in neurons is significantly stimulated in response to the metabolic acidification associated with an ischemic/hypoxic insult. Despite its function in H^+ extrusion and recovery of pH_i, increased NHE1 activity causes intracellular Na^+ and Ca^{2+} overload (in later discussion in section IV) which eventually leads to cell death.

4.2. NHE in ischemic glial damage

Recently, we have performed a series of studies on the role of NHE1 in ischemic astrocyte damage. We found that under nominally bicarbonate-free conditions NHE1 is the primary pH regulatory mechanism in cortical astrocytes. 2 h OGD and 2 min REOX cause pH_i to fall by 0.29 pH units (31). Either inhibition of NHE1 with HOE 642 or genetic ablation of NHE1 results in a further decrease of pH_i. Upon REOX, NHE1 activity is increased by ~ 1.8 fold in $NHE1^{+/+}$ astrocytes (31). In contrast, NHE1 activity is blocked in $NHE1^{+/+}$ astrocytes in the presence of HOE 642 or in $NHE1^{-/-}$ astrocytes. This OGD-induced increase in NHE1 activity depends on ERK1/2 signaling pathways (32). Phosphorylated ERK1/2 signals are increased in astrocytes during 1 h OGD as well as REOX. MEK inhibitor PD-98059 blocks OGD-mediated stimulation of NHE1 (32).

Increased NHE1 activity in astrocytes following OGD may result in an intracellular Na^+ overload that is substantially mediated through activation of the ERK 1/2 pathways (32). We observed that 2 h OGD followed by 1 h REOX triggers a ~5-fold increase in $[Na^+]_i$. OGD also results in astrocyte swelling by 26%. The OGD-induced increase in $[Na^+]_i$ and cell swelling are significantly reduced either with HOE 642 or in $NHE1^{-/-}$ astrocytes (31).

In a more severe model of in vitro ischemia in astrocytes, cultured astrocytes were superfused with a hypoxic, acidic, ion shifted ringers (HAIR) solutions at 37°C, a condition which mimics the ionic composition of the ischemic extracellular space (33,34). Exposure to HAIR causes a rapid decline in astrocyte pH_i that the cells failed to regulate. This is consistent with the finding that NHE1 activity is inhibited by low pH_o (35). Upon return to normal buffer, astrocytes rapidly alkalize and pH_i substantially overshoot the baseline suggesting a role for NHE1 (34). Using a similar model, we found that there is a large transient in $[Na^+]_i$ that plateaues ~ 5 times higher than baseline by 20 min REOX (18). However, when astrocytes are treated with 1 μM HOE 642 during REOX, the HAIR/REOX-induced $[Na^+]_i$ transient is abolished and $[Na^+]_i$ returns to a baseline (18). These experiments provide evidence that NHE1 is stimulated following severe hypoxic/ischemic conditions and responsible for accumulation of Na^+ in astrocytes.

Bondarenko et al. has reported that HAIR/REOX leads to ~ 40% cell death in astrocytes (34). The presence of the NHE1 blockers EIPA or HOE 694 during HAIR and REOX or during REOX alone significantly decreases HAIR-induced cell death (34). Moreover, following 2 h OGD, the PCr/Cr falls 46% of control in C6 and F98 glioma lines. When the NHE1 inhibitor HOE 642 is present during hypoxia the energy state of glioma cells is preserved and the PCr/Cr ratio only falls to 87% (36). Taken together, NHE1 plays an important role in regulation of astrocyte pH_i. Post-ischemic REOX triggers a significant elevation of NHE1 activity which perturbs Na^+ homeostasis and leads to astrocyte death.

4.3. NHE in focal ischemia

Substantial *in vivo* studies suggest that NHEs play an important role in the pathogenesis of neuronal ischemic damage. Inhibition of NHEs by various inhibitors or ablation of NHE1 gene expression shows neuroprotection in animal ischemic models. In our recent study, we examine in parallel the neuroprotective effects of the potent NHE1 inhibitor HOE 642 in $NHE1^{+/+}$ mice and knockdown of NHE1 expression in $NHE1^{+/-}$ mice. There is a marked decrease of infarct volume in HOE 642-treated $NHE1^{+/+}$ mice compared with non-treated $NHE1^{+/+}$ mice following MCAO [56.8 ± 25.9 vs. 84.8 ± 17.7 mm^3, respectively, $p < 0.05$, (30)]. Similar reduction of infarct volume is found in $NHE1^{+/-}$ mice which express < 50% of NHE1 protein [58.2 ± 11.8 mm^3, $p < 0.05$, (30)]. These studies firmly demonstrate the dominant role of NHE1 among other NHE isoforms in cerebral ischemic brain damage. This view is supported by the finding that NHE1 is the most abundant NHE isoform in the CNS (37).

SM-20220 (N-(aminoiminomethyl)-1-methyl-1H-indole-2-carboxamide methanesulfonate), a highly selective NHE inhibitor, given intravenously 1 h after MCAO significantly reduces the extent of cerebral edema and Na^+ content after 2 h ischemia and 4 h reperfusion and infarct volume after 22 h reperfusion (38). SM-20220 (1.0 mg/kg, iv) decreases infarct size in both transient and permanent MCAO models. Especially, there is a reduction of infarct size when the treatment is delayed for 5, 30, or 60 min after the onset of ischemia. This suggests that SM-20220 may be useful for stroke treatments (39). SM-20220-mediated protection was further evaluated after MCA thrombotic occlusion using a bolus injection of the drug just at the onset of the occlusion, followed immediately by 2.5 h continuous infusion (40). SM-20220 (39 microgram/kg) leads to ~ 50% decrease in infarct size at 72 h reperfusion.

Similar neuroprotection in cerebral ischemia is observed using other NHE inhibitors. Kitayama et al (41) reported reduction of infarct volume and brain edema with FR-183998, (5-(2,5-dichlorothiophen-3-yl)-3-[(2-dimethylaminoethyl)carbamoyl]benzoylguanidine dihydrochloride), another inhibitor of NHE. FR-183998 (1 mg/kg) administered intravenously prior to the MCAO reduces infarct volume by 29% after 72 h reperfusion. Sabiporide (3mg/kg, iv), N-carbamimidoyl-4-[4-(1H-pyrrol-2-ylcarbonyl)piperazin-1-yl]-3-(trifluoromethyl)benzamide, a highly specific NHE inhibitor with long-lasting inhibitory effects (42), decreases infarct size and edema volume at 24 h reperfusion not only in pre-ischemia treated but also in post-ischemia treated rats (43). Moreover, SM-20220

reduces the number of neutrophils in the ischemic hemisphere (40). The authors suggest that the reduction of infarction and edema might be due to the inhibition of neutrophils. Delayed accumulation of these cells in reactive zones around infarction is well established in human stroke autopsies (44) and represents a central feature of inflammation. SM-20220 attenuates leukocyte adhesion and migration in the mesenteric artery (45) and improves endothelial dysfunction (46).

NHE inhibitors not only reduce cell death and edema, but also preserve neurological functions in different ischemic injury animal models. Castellá et al (47) observe a rapid neurological recovery in Yorkshire-Duroc pigs receiving HOE 642 just at the onset of cooling which underwent femoral-jugular bypass and 90 min of deep hypothermic circulatory arrest at 19°C. The consciousness recovery time was also measured following a 30 min transient global cerebral ischemia in Mongolian gerbils. Intravenous administration of SM-20220 at 1.0 mg/kg significantly lowers the neurological score (McGraw's scale) at 2 h reperfusion, compared to the vehicle group ($p<0.01$) (48). This improvement in neurological deficit persists until 24 h reperfusion. Phillis et al (49) demonstrate that EIPA not only protects gerbil hippocampal neuron from ischemic injury but also significantly reduces the magnitude of the ischemia-induced increase in locomotor activity at both 24 h and 6 days reperfusion. Ischemic injury to the gerbil forebrain produces an increase in locomotor activity which is related to the degree of neuronal damage in the CA1 region of the hippocampus (50).

5. Na^+/Ca^{2+} EXCHANGER

The plasma membrane NCX is widely distributed in the central nervous system. NCX works bidirectionally depending on $[Na^+]_i$, $[Ca^{2+}]_i$ and plasma membrane potential. Normally, NCX extrudes 1 Ca^{2+} ion in exchange for an influx of 3 Na^+ ions (51). Under physiological conditions, the NCX is thought to primarily pump Ca^{2+} to the outside of the cell using the Na^+ concentration gradient across the cell membrane. In contrast, under special conditions in which Na^+ accumulates inside the cell, the NCX can transport Ca^{2+} influx (reverse mode-operation of NCX). There are 3 isoforms in the NCX family [NCX1, NCX2, and NCX3, (52)]. The NCX1 gene is expressed in several tissues, including brain, heart, skeletal muscle etc., while NCX2 and NCX3 are expressed mainly in the brain and skeletal muscle (53). The interested reader can refer to the recent and extensive review on NCX by Annunziato and colleagues (54).

5.1. Role of NCX in ischemic neuronal damage

It has been reported that reversal of NCX is an important contributor to the early increase in $[Ca^{2+}]_i$ caused by NMDA and non-NMDA receptor activation in rat cortical neurons (55). However, inhibition of the reverse mode of NCX with KB-R7943 does not reduce the Ca^{2+} load or neuronal cell death induced by prolonged glutamate receptor activation (55). In contrast, in rat cerebellar granule cells, a large part of NMDA-induced Ca^{2+} influx in depolarized and glucose-deprived cells is mediated by reversal of NCX. This high level of reversed NCX operation is maintained by a sustained Na^+ influx via NMDA channels and depolarization of the plasma membrane (56). However, when the cells are energized with glucose, Na^+/K^+ ATPase partially regenerates the Na^+ and K^+ concentration gradients which prevents reversal of NCX and most Ca^{2+} enters directly via NMDA channels (56). Additionally, Kiedrowski et al. show that inhibition of NCX with KB-R7943 is neuroprotective in glucose-deprived / depolarized primary cultures of forebrain neurons (57). These findings are consistent with our reports that NHE1 or NKCC1-mediated intracellular Na^+ accumulation following OGD/REOX is a prerequisite for reversal of NCX and Ca^{2+} overload (17,30,31,32).

Breder (58) investigated the effects of inhibiting NCX on neuronal damage in organotypic slice cultures. They found that KB-R7943 is neuroprotective when administered at 0.1 μM, but paradoxically exacerbates neuronal damage at higher concentrations. KB-R7943 also significantly reduces the OGD-induced increase in $[Ca^{2+}]_i$ in pyramidal neurons and generates significant neuroprotection in rat brain slice cultures after either 20 min or 40 min OGD followed by 1 h REOX (59). Tanaka (60) subjected hippocampal slices to 5 min OGD and observed a sharp rise in $[Ca^{2+}]_i$ in CA1 neurons. When the slices are reoxygenated, $[Ca^{2+}]_i$ gradually decreases towards baseline. The OGD-induced increase in $[Ca^{2+}]_i$ is significantly decreased by using the non-specific NCX inhibitor Ni^{2+}. However, the decrease in $[Ca^{2+}]_i$ during REOX is blocked by either Ni^{2+} or removal of extracellular sodium, suggesting a role for the forward-mode of NCX.

Although the physiological significance of tissue-specific isoforms NCX2 and NCX3 is unclear, Bano et al. (61) recently report that NCX3, but not NCX1 and NCX2, is cleaved in cerebellar granule cells (CGN) following glutamate-induced excitotoxicity. The delayed Ca^{2+} deregulation and death in CGNs are exacerbated when NCX3 is down-regulated by siRNA. This suggests that there are differential changes among the three NCX isoforms in response to excitotoxicity/ischemia and NCX3-driven neuronal Ca^{2+} extrusion is critical for CGN survival following glutamate-mediated damage.

5.2. NCX contributes to ischemic white matter and glial damage

The role of NCX exchange in axonal damage in central myelinated white matter tracts is well documented (62). During anoxia or trauma, intra-axonal Na^+ increases via activation of voltage-gated Na^+ channels, which promotes NCX reversal and leads to a detrimental Ca^{2+} overload. The compound action potentials of rat spinal dorsal columns are preserved following either 60 min anoxic perfusion or compression injury when either bepridil or KB-R79473 is present (63). In studies using spinal root myelinated axons, bepredil could protect from morphological degradation induced by the free radical nitric oxide (64). These findings suggest that Ca^{2+} overload via altered NCX activity is an important cause of excitotoxic and ischemic white matter damage.

Several reports also indicate the role of NCX in disruption of Ca^{2+} homeostasis in astrocytes following hypoxia/ischemia. Reversal of NCX has been demonstrated in astrocytes and can be blocked by ~ 90% with 3 µM KB-R7943 (17). Interestingly, ~85 % of the REOX-triggered Ca^{2+} rise following 5 min HAIR is abolished with 3 µM KB-R7943 (18). The data suggest that Ca^{2+} entry during REOX is largely via reversal of NCX in astrocytes. This finding is consistent with our thermodynamic analysis that NCX would operate in the reverse mode and mediate Ca^{2+} influx when $[Na^+]_i$ in astrocytes is increased above 25 mM (32). Peak values of $[Na^+]_i$ reach 70-80 mM during REOX either following OGD or HAIR, a condition which favors the reverse operation of NCX (18). This view is supported by several recent reports. For example, inhibition of NCX with 100 nM KB-R7943 significantly attenuates the rise in intracellular Ca^{2+} in response to severe mechanical strain injuries in rat cortical astrocytes (65). The strain injury leads to a rapid rise in $[Na^+]_i$ in astrocytes that is sustained for ~ 50 min (65). Moreover, transient elevation of $[Ca^{2+}]_i$ following 25-30 min HAIR is blocked by the NCX inhibitor KB-R7943 (34).

5.3. NCX in cerebral ischemia

However, the role of NCX in cerebral ischemia is conflicting. NCX reversal selective inhibitor SEA0400 is neuroprotective in rat cerebral cortex and striatum after a transient focal ischemia (66). SEA0400 (3 mg/kg bolus, iv. plus 2 h continuous infusion at 3 mg/kg/h) reduces infarct volume >50% in cortex. In addition, topically applying KB-R7943 onto rat cerebral cortex prior to and during ischemia in rats attenuates free fatty acid (FFA) efflux which may be by reducing the activity of a specific isoform of phospholipase A2 (PLA_2) (67). FFA accumulation plays a role in the severity of cerebral ischemic damage (68). KB-R7943 (10 microg/kg , icv) infused by an osmotic minipump for 24 h from the beginning of pMCAO causes a reduction of the ischemic volume (69). However, in addition to NCX inhibition, KB-R7943 might protect cells partly through its remarkable and prolonged hypothermic effect in brains (69).

In contrast, Tortiglione et al. (70) report that during permanent ischemic injury, increased activation of NCX in a reversed mode may be neuroprotective, whereas its pharmacological blockade can exacerbate brain injury. They found all NCX inhibitors, such as tyrosine-6 glycosylated form of the exchanger inhibitory peptide (GLU-XIP), benzamil derivative (CB-DMB) and bepridil cause a worsening of the brain infarct lesion; however, $FeCl_3$, a forward NCX activator, is able to reduce the extension of infarct volume. These results are interpreted by the investigators as follows. In the penumbral region, where the Na^+/K^+-ATPase activity is still preserved, NCX may likely operate in a forward mode, and therefore, the inhibition of NCX in this area may lead to Ca^{2+}-induced cell injury by reducing the extrusion of Ca^{2+}. In contrast, as the Na^+/K^+-ATPase activity is reduced in the ischemic core region, accumulation of Na^+ may lead to NCX in the reverse mode. The inhibition of NCX further worsens the necrotic lesion of the surviving glial and neuronal cell as the loading of intracellular Na^+ increase (69,70). However, it is paradoxical as to why reduced Ca^{2+} influx via inhibiting the reversal of NCX in the latter condition is not neuroprotective.

NCX1, NCX2, and NCX3 isoforms are differentially expressed in distinct regions of the CNS and may not be involved in the physiological and pathophysiological functions in the same manner (53). Boscia et al (71) found that the pattern of post-ischemic NCX1, NCX2 and NCX3 gene expression varies depending on the region involved in the insult. In the ischemic core, all three NCX transcripts are down-regulated. In the peri-infarct area, NCX2 mRNA is down-regulated, whereas NCX3 mRNA is significantly up-regulated. In remote non-ischemic brain regions, both NCX1 and NCX3 are up-regulated, whereas NCX2 signal is strongly decreased. Knockout of NCX1, NCX2 and NCX3 genes with anti-sense approaches show that NCX1 and NCX3 play a major protective role in the development of permanent focal cerebral ischemia (73). After MCAO ischemia and 24 h reperfusion NCX3 is cleaved into two fragments of about 58-60 kDa in both cortex and striatum. NCX1 is only partly degraded and NCX2 is not cleaved (61).

6. SUMMARY

In summary, results from both *in vitro* and *in vivo* experimental studies suggest that NKCC1, NHE, and NCX play a role in ischemic cell damage. Although NCX1-mediated Ca^{2+} overload in myocardial ischemia/reperfusion is known to cause myocardial cell injury (72), studies on NCX in *in vivo* cerebral ischemic damage show conflicting results. This suggests that functions of NCX in the pathogenesis of cerebral ischemia are complex. Further study is needed to clarify the exact role of each NCX isoform in cerebral ischemia. Isoform specific inhibitors are needed. These ion transport proteins are potential targets to reduce or prevent ischemia-mediated loss of ion homeostasis.

7. ACKNOWLEDGMENTS:

This work was supported in part by an NIH grants R01NS38118, RO1NS48216 and AHA EIA 0540154 (D. Sun)

8. REFERENCES

1.Siesjö, B. K.: Pathophysiology and treatment of focal cerebral ischemia. Part I: Pathophysiology. *J Neurosurg* 77, 169-184 (1992)

2. Lipton, P.: Ischemic cell death in brain neurons. *Physiol Rev* 79, 1431-1568 (1999)

3. Grassl, S.: Mechanisms of Carrier-Mediated Transport: Facilitated Diffusion, Cotransport, and Countertransport. In: Cell Physiology Sourcebook Eds: Sperelakis N Academic Press, San Diego, Ca, 3, 249-260 (2001)

4. Russell, J. M.: Sodium-potassium-chloride cotransport. *Physiol Rev* 80, 211-276 (2000)

5. Plotkin, M. D., M. R. Kaplan, L. N. Peterson, S. R. Gullans, S. C. Hebert, & E. Delpire: Expression of the

Na(+)-K(+)-2Cl- cotransporter BSC2 in the nervous system. *Am J Physiol* 272, C173-C183 (1997)
6. Rothman, S. M.: The neurotoxicity of excitatory amino acids is produced by passive chloride influx. *J Neurosci* 5, 1483-1489 (1985)
7. Choi, D. W., M. Maulucci-Gedde, & A. R. Kriegstein: Glutamate neurotoxicity in cortical cell culture. *J Neurosci* 7, 357-368 (1987)
8. Schomberg, S. L., G. Su, R. A. Haworth, & D. Sun: Stimulation of Na-K-2Cl cotransporter in neurons by activation of Non-NMDA ionotropic receptor and group-I mGluRs. *J Neurophysiol* 85, 2563-2575 (2001)
9. Beck, J., B. Lenart, D. B. Kintner, & D. Sun: Na-K-Cl cotransporter contributes to glutamate-mediated excitotoxicity. *J Neurosci* 23, 5061-5068 (2003)
10. Gwag, B. J., J. Y. Koh, J. A. DeMaro, H. S. Ying, M. Jacquin, & D. W. Choi: Slowly triggered excitotoxicity occurs by necrosis in cortical cultures. *Neuroscience* 77, 393-401 (1997)
11. Chen, H., J. Luo, D. B. Kintner, G. E. Shull, & D. Sun: Na^+-dependent chloride transporter (NKCC1)-null mice exhibit less gray and white matter damage after focal cerebral ischemia. *J Cereb Blood Flow Metab* 25, 54-66 (2005)
12. Yan, Y. P., R. J. Dempsey, & D. Sun: Expression of Na^+-K^+-Cl^- cotransporter in rat brain during development and its localization in mature astrocytes. *Brain Res* 911, 43-55 (2001)
13. Wang, H., Y. Yan, D. B. Kintner, C. Lytle, & D. Sun: GABA-mediated trophic effect on oligodendrocytes requires Na-K-2Cl cotransport activity. *J Neurophysiol* 90, 1257-1265 (2003)
14. Su, G., D. B. Kintner, & D. Sun: Contribution of Na^+-K^+-Cl^- cotransporter to high $[K^+]_o$-induced swelling and excitatory amino acid release in rat astrocytes. *Am J Physiol Cell Physiol* 282, C1136-C1146 (2002)
15. Su, G., D. B. Kintner, M. Flagella, G. E. Shull, & D. Sun: Astrocytes from Na^+-K^+-Cl^- cotransporter-null mice exhibit absence of swelling and decrease in EAA release. *Am J Physiol Cell Physiol* 282, C1147-C1160 (2002)
17. Lenart, B., D. B. Kintner, G. E. Shull, & D. Sun: Na-K-Cl cotransporter-mediated intracellular Na^+ accumulation affects Ca^{2+} signaling in astrocytes in an in vitro ischemic model. *J Neurosci* 24, 9585-9597 (2004)
18. Kintner, D. B., J. Luo, J. Gerdts, A. J. Ballard, G. E. Shull, & D. Sun: Na^+-K^+-Cl^- cotransport and Na^+/Ca^{2+} exchange in astrocyte mitochondrial dysfunction in response to in vitro ischemia. *J Neurosci* (2006)
19. Yan, Y. P., R. J. Dempsey, & D. Sun: Na^+-K^+-Cl^- cotransporter in rat focal cerebral ischemia. *J Cereb Blood Flow Metab* 21, 711-721 (2001)
20. O'Donnell, M. E., L. Tran, T. I. Lam, X. B. Liu, & S. E. Anderson: Bumetanide inhibition of the blood-brain barrier Na-K-Cl cotransporter reduces edema formation in the rat middle cerebral artery occlusion model of stroke. *J Cereb Blood Flow Metab* 24, 1046-1056 (2004)
21. Yam, P. S., T. Takasago, D. Dewar, D. I. Graham, & J. McCulloch: Amyloid precursor protein accumulates in white matter at the margin of a focal ischaemic lesion. *Brain Res* 760, 150-157 (1997)
22. Brett, C. L., M. Donowitz, & R. Rao: Evolutionary origins of eukaryotic sodium/proton exchangers. *Am J Physiol Cell Physiol* 288, C223-C239 (2005)
23. Orlowski, J. & S. Grinstein: Diversity of the mammalian sodium/proton exchanger SLC9 gene family. *Pflugers Arch* 447, 549-565 (2004)
24. Ma, E. & G. G. Haddad: Expression and localization of Na^+/H^+ exchangers in rat central nervous system. *Neuroscience* 79, 591-603 (1997)
25. Putney, L. K., S. P. Denker, & D. L. Barber: The changing face of the Na^+/H^+ exchanger, NHE1: structure, regulation, and cellular actions. *Annu Rev Pharmacol Toxicol* 42, 527-552 (2002)
26. Yao, H., X. Q. Gu, R. M. Douglas, & G. G. Haddad: Role of Na^+/H^+ exchanger during O_2 deprivation in mouse CA1 neurons. *Am J Physiol Cell Physiol* 281, C1205-C1210 (2001)
27. Sheldon, C. & J. Church: Intracellular pH response to anoxia in acutely dissociated adult rat hippocampal CA1 neurons. *J Neurophysiol* 87, 2209-2224 (2002)
28. Vornov, J. J., A. G. Thomas, & D. Jo: Protective effects of extracellular acidosis and blockade of sodium/hydrogen ion exchange during recovery from metabolic inhibition in neuronal tissue culture. *J Neurochem* 67, 2379-2389 (1996)
29. Jorgensen, N. K., S. F. Petersen, I. Damgaard, A. Schousboe, & E. K. Hoffmann: Increases in $[Ca^{2+}]_i$ and changes in intracellular pH during chemical anoxia in mouse neocortical neurons in primary culture. *J Neurosci Res* 56, 358-370 (1999)
30. Luo, J., H. Chen, D. B. Kintner, G. E. Shull, & D. Sun: Decreased neuronal death in Na^+/H^+ exchanger isoform 1-null mice after in vitro and in vivo ischemia. *J Neurosci* 25, 11256-11268 (2005)
31. Kintner, D. B., G. Su, B. Lenart, A. J. Ballard, J. W. Meyer, L. L. Ng, G. E. Shull, & D. Sun: Increased tolerance to oxygen and glucose deprivation in astrocytes from Na^+/H^+ exchanger isoform 1 null mice. *Am J Physiol Cell Physiol* 287, C12-C21 (2004)
32. Kintner, D. B., A. Look, G. E. Shull, & D. Sun: Stimulation of astrocyte Na^+/H^+ exchange activity in response to in vitro ischemia in part depends on activation of extracellular signal-regulatory kinase. *Am J Physiol Cell Physiol* 289, C934-C945 (2005)
33. Bondarenko, A. & M. Chesler: Rapid astrocyte death induced by transient hypoxia, acidosis, and extracellular ion shifts. *Glia* 34, 134-142 (2001)
34. Bondarenko, A., N. Svichar, & M. Chesler: Role of Na^+-H^+ and Na^+-Ca^{2+} exchange in hypoxia-related acute astrocyte death. *Glia* 49, 143-152 (2005)
35. Mellergard, P. E., Y. B. Ouyang, & B. K. Siesjo: The regulation of intracellular pH in cultured astrocytes and neuroblastoma cells, and its dependence on extracellular pH in a HCO_3^- free solution. *Can J Physiol Pharmacol* 70 Suppl, S293-S300 (1992)
36. Glunde, K., H. Dussmann, H. P. Juretschke, & D. Leibfritz: Na^+/H^+ exchange subtype 1 inhibition during extracellular acidification and hypoxia in glioma cells. *J Neurochem* 80, 36-44 (2002)
37. Douglas, R. M., B. M. Schmitt, Y. Xia, M. O. Bevensee, D. Biemesderfer, W. F. Boron, & G. G. Haddad: Sodium-hydrogen exchangers and sodium-bicarbonate co-transporters: ontogeny of protein expression in the rat brain. *Neuroscience* 102, 217-228 (2001)

38. Kuribayashi, Y., N. Itoh, M. Kitano, & N. Ohashi: Cerebroprotective properties of SM-20220, a potent Na^+/H^+ exchange inhibitor, in transient cerebral ischemia in rats. *Eur J Pharmacol* 383, 163-168 (1999)
39. Kuribayashi, Y., N. Horikawa, N. Itoh, M. Kitano, & N. Ohashi: Delayed treatment of Na+/H+ exchange inhibitor SM-20220 reduces infarct size in both transient and permanent middle cerebral artery occlusion in rats. *Int J Tissue React* 21, 29-33 (1999)
40. Suzuki, Y., Y. Matsumoto, Y. Ikeda, K. Kondo, N. Ohashi, & K. Umemura: SM-20220, a Na(+)/H(+) exchanger inhibitor: effects on ischemic brain damage through edema and neutrophil accumulation in a rat middle cerebral artery occlusion model. *Brain Res* 945, 242-248 (2002)
41. Kitayama, J., T. Kitazono, H. Yao, H. Ooboshi, H. Takaba, T. Ago, M. Fujishima, & S. Ibayashi: Inhibition of Na^+/H^+ exchanger reduces infarct volume of focal cerebral ischemia in rats. *Brain Res* 922, 223-228 (2001)
42. Touret, N., V. Tanneur, H. Godart, R. Seidler, N. Taki, E. Burger, J. Dammgen, & L. Counillon: Characterization of sabiporide, a new specific NHE-1 inhibitor exhibiting slow dissociation kinetics and cardioprotective effects. *Eur J Pharmacol* 459, 151-158 (2003)
43. Park, H. S., B. K. Lee, S. Park, S. U. Kim, S. H. Lee, E. J. Baik, S. Lee, K. Y. Yi, S. E. Yoo, C. H. Moon, & Y. S. Jung: Effects of sabiporide, a specific Na^+/H^+ exchanger inhibitor, on neuronal cell death and brain ischemia. *Brain Res* 1061, 67-71 (2005)
44. Kochanek, P. M. & J. M. Hallenbeck: Polymorphonuclear leukocytes and monocytes/macrophages in the pathogenesis of cerebral ischemia and stroke. *Stroke* 23, 1367-1379 (1992)
45. Horikawa, N., M. Nishioka, N. Itoh, Y. Kuribayashi, K. Matsui, & N. Ohashi: The Na^+/H^+ exchanger SM-20220 attenuates ischemic injury in in vitro and in vivo models. *Pharmacology* 63, 76-81 (2001)
46. Horikawa, N., Y. Kuribayashi, N. Itoh, M. Nishioka, K. Matsui, N. Kawamura, & N. Ohashi: Na^+/H^+ exchange inhibitor SM-20220 improves endothelial dysfunction induced by ischemia-reperfusion. *Jpn J Pharmacol* 85, 271-277 (2001)
47. Castellá, M., G. D. Buckberg, & Z. Tan: Neurologic preservation by Na^+-H^+ exchange inhibition prior to 90 minutes of hypothermic circulatory arrest. *Ann Thorac Surg* 79, 646-654 (2005)
48. Kuribayashi, Y., N. Itoh, N. Horikawa, & N. Ohashi: SM-20220, a potent Na+/H+ exchange inhibitor, improves consciousness recovery and neurological outcome following transient cerebral ischaemia in gerbils. *J Pharm Pharmacol* 52, 441-444 (2000)
49. Phillis, J. W., A. Y. Estevez, L. L. Guyot, & M. H. O'Regan: 5-(N-Ethyl-N-isopropyl)-amiloride, an Na^+-H^+ exchange inhibitor, protects gerbil hippocampal neurons from ischemic injury. *Brain Res* 839, 199-202 (1999)
50. Gerhardt, S. C. & C. A. Boast: Motor activity changes following cerebral ischemia in gerbils are correlated with the degree of neuronal degeneration in hippocampus. *Behav Neurosci* 102, 301-3, 328 (1988)
51. Kang, T. M. & D. W. Hilgemann: Multiple transport modes of the cardiac Na^+/Ca^{2+} exchanger. *Nature* 427, 544-548 (2004)
52. Nicoll, D. A., M. Ottolia, & K. D. Philipson: Toward a topological model of the NCX1 exchanger. *Ann N Y Acad Sci* 976, 11-18 (2002)
53. Lee, S. L., A. S. Yu, & J. Lytton: Tissue-specific expression of Na(+)-Ca2+ exchanger isoforms. *J Biol Chem* 269, 14849-14852 (1994)
54. Annunziato, L., G. Pignataro, & G. F. Di Renzo: Pharmacology of brain Na^+/Ca^{2+} exchanger: from molecular biology to therapeutic perspectives. *Pharmacol Rev* 56, 633-654 (2004)
55. Hoyt, K. R., S. R. Arden, E. Aizenman, & I. J. Reynolds: Reverse Na^+/Ca^{2+} exchange contributes to glutamate-induced intracellular Ca^{2+} concentration increases in cultured rat forebrain neurons. *Mol Pharmacol* 53, 742-749 (1998)
56. Czyz, A. & L. Kiedrowski: In depolarized and glucose-deprived neurons, Na^+ influx reverses plasmalemmal K^+-dependent and K^+-independent Na^+/Ca^{2+} exchangers and contributes to NMDA excitotoxicity. *J Neurochem* 83, 1321-1328 (2002)
57. Kiedrowski, L., A. Czyz, G. Baranauskas, X. F. Li, & J. Lytton: Differential contribution of plasmalemmal Na/Ca exchange isoforms to sodium-dependent calcium influx and NMDA excitotoxicity in depolarized neurons. *J Neurochem* 90, 117-128 (2004)
58. Breder, J., C. F. Sabelhaus, T. Opitz, K. G. Reymann, & U. H. Schroder: Inhibition of different pathways influencing Na(+) homeostasis protects organotypic hippocampal slice cultures from hypoxic/hypoglycemic injury. *Neuropharmacology* 39, 1779-1787 (2000)
59. Martinez-Sanchez, M., F. Striggow, U. H. Schroder, S. Kahlert, K. G. Reymann, & G. Reiser: Na(+) and Ca(2+) homeostasis pathways, cell death and protection after oxygen-glucose-deprivation in organotypic hippocampal slice cultures. *Neuroscience* 128, 729-740 (2004)
60. Tanaka, H., K. Nishimaru, T. Aikawa, W. Hirayama, Y. Tanaka, & K. Shigenobu: Effect of SEA0400, a novel inhibitor of sodium-calcium exchanger, on myocardial ionic currents. *Br J Pharmacol* 135, 1096-1100 (2002)
61. Bano, D., K. W. Young, C. J. Guerin, R. Lefeuvre, N. J. Rothwell, L. Naldini, R. Rizzuto, E. Carafoli, & P. Nicotera: Cleavage of the plasma membrane Na^+/Ca^{2+} exchanger in excitotoxicity. *Cell* 120, 275-285 (2005)
62. Stys, P. K.: General mechanisms of axonal damage and its prevention. *J Neurol Sci* 233, 3-13 (2005)
63. Li, S., Q. Jiang, & P. K. Stys: Important role of reverse Na^+-Ca^{2+} exchange in spinal cord white matter injury at physiological temperature. *J Neurophysiol* 84, 1116-1119 (2000)
64. Kapoor, R., M. Davies, P. A. Blaker, S. M. Hall, & K. J. Smith: Blockers of sodium and calcium entry protect axons from nitric oxide-mediated degeneration. *Ann Neurol* 53, 174-180 (2003)
65. Floyd, C. L., F. A. Gorin, & B. G. Lyeth: Mechanical strain injury increases intracellular sodium and reverses Na^+/Ca^{2+} exchange in cortical astrocytes. *Glia* 51, 35-46 (2005)
66. Matsuda, T., N. Arakawa, K. Takuma, Y. Kishida, Y. Kawasaki, M. Sakaue, K. Takahashi, T. Takahashi, T. Suzuki, T. Ota, A. Hamano-Takahashi, M. Onishi, Y. Tanaka, K. Kameo, & A. Baba: SEA0400, a novel and selective inhibitor of the Na^+-Ca^{2+} exchanger, attenuates

reperfusion injury in the in vitro and in vivo cerebral ischemic models. *J Pharmacol Exp Ther* 298, 249-256 (2001)
67. Pilitsis, J. G., F. G. Diaz, M. H. O'Regan, & J. W. Phillis: Inhibition of Na(+)/Ca(2+) exchange by KB-R7943, a novel selective antagonist, attenuates phosphoethanolamine and free fatty acid efflux in rat cerebral cortex during ischemia-reperfusion injury. *Brain Res* 916, 192-198 (2001)
68. Katayama, Y., T. Kawamata, T. Maeda, & T. Tsubokawa: Free fatty acid liberation and cellular swelling during cerebral ischemia: the role of excitatory amino acids. *Acta Neurochir Suppl (Wien)* 60, 242-245 (1994)
69. Pignataro, G., A. Tortiglione, A. Scorziello, L. Giaccio, A. Secondo, B. Severino, V. Santagada, G. Caliendo, S. Amoroso, G. Di Renzo, & L. Annunziato: Evidence for a protective role played by the Na^+/Ca^{2+} exchanger in cerebral ischemia induced by middle cerebral artery occlusion in male rats. *Neuropharmacology* 46, 439-448 (2004)
70. Tortiglione, A., G. Pignataro, M. Minale, A. Secondo, A. Scorziello, G. F. Di Renzo, S. Amoroso, G. Caliendo, V. Santagada, & L. Annunziato: Na^+/Ca^{2+} exchanger in Na^+ efflux-Ca^{2+} influx mode of operation exerts a neuroprotective role in cellular models of in vitro anoxia and in vivo cerebral ischemia. *Ann N Y Acad Sci* 976, 408-412 (2002)
71. Boscia, F., R. Gala, G. Pignataro, A. de Bartolomeis, M. Cicale, A. Ambesi-Impiombato, G. Di Renzo, & L. Annunziato: Permanent focal brain ischemia induces isoform-dependent changes in the pattern of Na(+)/Ca(2+) exchanger gene expression in the ischemic core, periinfarct area, and intact brain regions. *J Cereb Blood Flow Metab* (2005)
72. Avkiran, M.: Protection of the ischaemic myocardium by Na^+/H^+ exchange inhibitors: potential mechanisms of action. *Basic Res Cardiol* 96, 306-311 (2001)
73. Pignataro G, Gala R, BS; Cuomo O, Tortiglione A; Giaccio L; Castaldo P; Sirabella R; Matrone C; Canitano A; Amoroso S; Di Renzo G, Annunziato L. Two Sodium/Calcium Exchanger Gene Products, NCX1 and NCX3, Play a Major Role in the Development of Permanent Focal Cerebral Ischemia. *Stroke*, 35: 2566-70 (2004).
74. Xiong ZG, Zhu XM, Chu XP, Minami M, Hey J, Wei WL, MacDonald JF, Wemmie JA, Price MP, Welsh MJ, Simon RP. Neuroprotection in ischemia: blocking calcium-permeable acid-sensing ion channels. *Cell.* 118(6):687-98 (2004)

Key Words: intracellular Ca^{2+}, Na^+/H^+ exchange, Na^+ influx, $Na^+/Ca2^+$ exchange, Bumetanide, Chloride Transport

Send correspondence to: Dandan Sun, M.D., PhD., Department of Neurological Surgery, University of Wisconsin Medical Center, H4/332 Clinical Sciences Center, 600 Highland Ave., Madison, WI 53792, Tel: 608-263-4060, Fax: 608-263-1409, E-mail: sun@neurosurg.wisc.edu