

Special Focus: Molecular and Cellular Events Controlling Neuronal and Brain Function and Dysfunction

Ion transporters and ischemic mitochondrial dysfunction

Yan Liu,^{1,2} Xiang-jun Liu² and Dandan Sun^{1,*}

¹Department of Neurological Surgery; University of Wisconsin School of Medicine and Public Health; Madison, WI USA; ²Department of Biological Sciences and Biotechnology; Institute of Biomedical Informatics; School of Medicine; Tsinghua University; Beijing, China

Abbreviation: AMPA, alphaamino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; Ca^{2+}_m , mitochondrial Ca^{2+} ; $\text{Ca}^{2+}_{\text{cyt}}$, cytosolic free Ca^{2+} ; $[\text{Ca}^{2+}]_{\text{cyt}}$, cytosolic Ca^{2+} concentration; Cyt. C, cytochrome *c*; CTZ, cyclothiazide; HAIR, a hypoxic, acidic, ion-shifted ringer buffer; InsP(3)R, inositol 1,4,5-triphosphate receptor; $\text{Na}^{+}_{\text{cyt}}$, cytoplasmic Na^{+} ; $[\text{Na}^{+}]_{\text{cyt}}$, cytosolic Na^{+} concentration; NCX, $\text{Na}^{+}/\text{Ca}^{2+}$ exchanger; NCX_{rev}, reverse-mode operation of NCX; NKCC, Na^{+} -dependent chloride transporter; NMDA, N-methyl-D-aspartic acid; OGD, oxygen-glucose deprivation; PTP, permeability transition pore; REOX, reoxygenation; Ψ_m , mitochondrial membrane potential

Key words: ischemia, intracellular Ca^{2+} dysregulation, changes of mitochondrial Ca^{2+} , cytochrome *c*, apoptosis

Ischemia-induced ionic imbalance leads to the activation of numerous events including mitochondrial dysfunction and eventual cell death. Dysregulation of mitochondrial Ca^{2+} (Ca^{2+}_m) plays a critical role in cell damage under pathological conditions including traumatic brain injury and stroke. High Ca^{2+}_m levels can induce the persistent opening of the mitochondrial permeability transition pore and trigger mitochondrial membrane depolarization, Ca^{2+} release, cessation of oxidative phosphorylation, matrix swelling and eventually outer membrane rupture with release of cytochrome *c* and other apoptogenic proteins. Thus, the dysregulation of mitochondrial Ca^{2+} homeostasis is now recognized to play a crucial role in triggering mitochondrial dysfunction and subsequent apoptosis. Recent studies show that some secondary active transport proteins, such as Na^{+} -dependent chloride transporter and $\text{Na}^{+}/\text{Ca}^{2+}$ exchanger, contribute to ischemia-induced dissipation of ion homeostasis including Ca^{2+}_m .

Introduction

Ionic imbalance plays a critical role in pathogenesis of ischemic cell damage. Ischemia-induced perturbation of ion homeostasis leads to intracellular accumulation of Ca^{2+} and Na^{+} which results in the subsequent activation of numerous events including mitochondrial dysfunction and eventual cell death. Structural, biochemical and functional abnormalities of mitochondria are widely believed to contribute to ischemic or hypoxic cell injury.¹ Many lines of evidences suggest that Ca^{2+} is a key regulator of mitochondrial function.^{2,3} Mitochondrial Ca^{2+} overload triggers the mitochondrial death pathway, which features the loss of mitochondrial membrane potential (Ψ_m), the opening of the mitochondrial permeability tran-

sition pore (PTP), release of Cytochrome *c* (Cyt. C), and enhanced generation of reactive oxygen species.⁴⁻⁶

This review will highlight recent studies reflecting the role of secondary active transport proteins in ischemia-induced dissipation of ion homeostasis and subsequent mitochondrial dysfunction. Secondary active ion transport proteins such as the Na^{+} -dependent chloride transporter (NKCC) and the $\text{Na}^{+}/\text{Ca}^{2+}$ exchanger (NCX) do not use energy stored in ATP directly, but derive their energy from the combined electrochemical gradients generated by $\text{Na}^{+}/\text{K}^{+}$ -ATPase and Ca^{2+} -ATPase. Secondary active ion transport proteins are important in maintaining steady-state intracellular ionic concentrations. Results from both in vitro and in vivo experimental studies suggest that these ion transport proteins are involved in ischemia-mediated loss of ion homeostasis, leading to mitochondrial dysfunction.⁷⁻¹¹ Therefore, they may be important targets for therapeutic intervention.

Mitochondrial Ca^{2+} Imbalance and Ischemic Mitochondrial Dysfunction

Mitochondrial Ca^{2+} imbalance. Calcium is one of the most prominent intracellular messenger molecules, and plays a vital role in the physiology and biochemistry of cells, particularly in signal transduction pathways.¹² Cytosolic free Ca^{2+} ($\text{Ca}^{2+}_{\text{cyt}}$) is maintained at roughly 100 nM while the extracellular milieu generally has a $[\text{Ca}^{2+}]$ of over 1 mM under physiological conditions. Loss of Ca^{2+} homeostasis, often in the form of an increase in cytoplasmic Ca^{2+} , leads to multiple destructive processes such as the activation of proteases, lipases, nucleases, nitric oxide synthases, protein kinases and eventual cell death.^{4,12}

Mitochondria play a central role in cellular metabolism and are responsible for cellular respiration that generates ATP from ADP and inorganic phosphate. Under physiological conditions, Ca^{2+}_m increases to buffer the amplitude of the $\text{Ca}^{2+}_{\text{cyt}}$ rise. Ca^{2+}_m is typically in the range of 0.2–3 mM, which is ideal for the activation of Ca^{2+} -dependent enzymes of the Krebs cycle. Increases of Ca^{2+}_m can be compensated by mitochondrial Ca^{2+} efflux mechanisms such as NCX and matrix Ca^{2+} buffering.³ Thus mitochondria accumulate

*Correspondence to: Dandan Sun; Department of Neurological Surgery; University of Wisconsin; T513 Waisman Center; 1500 Highland Ave.; Madison, WI 53705 USA; Tel.: 608.263.4060; Fax: 608.263.1409; Email: sun@neurosurg.wisc.edu

Submitted: 10/08/08; Accepted: 12/02/08

Previously published online as a *Cell Adhesion & Migration* E-publication: <http://www.landesbioscience.com/journals/celladhesion/article/7516>

Ca^{2+} and efficiently control the spatial and temporal shape of cellular Ca^{2+} signals, yet this situation exposes them to the hazards of Ca^{2+} overload.^{3,13} During anoxia/ischemia, oxidative phosphorylation is inhibited, triggering a rapid decline in ATP production and initiating multiple destructive processes.¹⁴ These changes include the compromising of ionic homeostasis, activation of glycolysis and intracellular acidosis, degradation of phospholipids and an increase in the plasma membrane permeability to Na^+ and Ca^{2+} .^{14,15} Elevated cytoplasmic Na^+ (Na^+_{cyt}) during ischemia will favor the reverse-mode operation of NCX (NCX_{rev}), causing $\text{Ca}^{2+}_{\text{cyt}}$ levels to further increase.¹⁶ In the presence of ATP, Mg^{2+} and inorganic phosphate, respiring mitochondria are able to accumulate large amounts of Ca^{2+} via the mitochondrial Ca^{2+} uniporter and/or the rapid uptake mode.¹⁷ However, during ischemia when ATP is decreased, mitochondria would not be able to accumulate Ca^{2+} . Upon reperfusion, increases in $\text{Ca}^{2+}_{\text{cyt}}$ or the release of Ca^{2+} from the endoplasmic reticulum invariably induce $\text{Ca}^{2+}_{\text{m}}$ uptake which helps in reestablishing physiological $\text{Ca}^{2+}_{\text{cyt}}$ levels.^{2,15}

Sustained increases in $\text{Ca}^{2+}_{\text{m}}$ will initiate several death factors. The rapid uptake of Ca^{2+} by mitochondria stimulates the Ca^{2+} -sensitive matrix dehydrogenases, which are key sites of NADH production for the respiratory chain and thereby for stimulation of mitochondrial energy metabolism.² A high concentration of mitochondria Ca^{2+} can also induce the opening of the PTP, a high conductance inner membrane channel which consists of the voltage-dependent anion channel, the adenine nucleotide translocator and the cyclophilin D, as well as several other proteins.^{18–20} Activation of PTP triggers a cascade of events during cellular damage.

Ischemic mitochondrial dysfunction. PTP opening contributes significantly to mitochondrial dysfunction. In particular it can dramatically increase the mitochondrial membrane permeability, allowing passage of solutes with molecular masses up to 1,500 Da. Persistent PTP opening is followed by depolarization of mitochondria leading to Ca^{2+} release, cessation of oxidative phosphorylation, matrix swelling with inner membrane remodeling and eventual outer membrane rupture with release of Cyt. C and other apoptogenic proteins.^{3,21,22} Released Cyt. C then binds to apoptotic protease activating factor-1, which recruits and activates caspase-9 to form the apoptosome. Caspase-9 activation results in the cleavage and activation of caspases-3 and -7, initiating a proteolytic cascade that ultimately results in cell death.²³ There are a number of factors that lower the threshold for Ca^{2+} induced mitochondrial permeability transition, including inorganic phosphate, prooxidants that oxidize membrane SH-groups, oxidation of NAD(P)H and GSH.¹⁵ On the other hand, a protective effect is exerted by Mg^{2+} , ADP (and ATP), some antioxidants, carnitine, decrease in pH and cyclosporin A that binds to cyclophilin.¹⁵

Members of the Bcl-2 family of proteins, notably Bax and Bak, can also trigger the permeabilization of the outer mitochondrial membrane by integrating into it as oligomers. This process is stimulated by t-Bid and other BH3-only proteins,²⁴ while inhibited by anti-apoptotic family members, Bcl-2 and Bcl-X_L.²⁵ Mice deficient in both Bax and Bak are resistant to most apoptotic stimuli, providing a strong support for the role of the mitochondrial pathway in apoptosis signaling.^{2,26}

The release of Cyt. C to cytoplasm is one of the key events of mitochondrial dysfunction. In healthy cells, Cyt. C is normally

bound to the inner mitochondrial membrane by its association with the anionic phospholipid cardiolipin, where it is predominantly located. The dissociation of Cyt. C from cardiolipin leads to the peroxidation of cardiolipin^{27,28} and decreases its binding affinity to haemoprotein. This is a crucial first step for Cyt. C release into the cytosol and for the induction of apoptosis.^{2,27} In addition, Ca^{2+} can bind to cardiolipin in the inner mitochondrial membrane, leading to decreased lipid mobility, formation of cardiolipin-enriched domains and protein aggregation.²⁹ In turn, this rearrangement leads to increased production of reactive oxygen species by the respiratory chain, which promotes the oxidation of membrane phospholipids and proteins resulting in an increase in membrane permeability. In fact, the Ca^{2+} -cardiolipin interaction might be an early and crucial step in the sequence of events by which Ca^{2+} triggers mitochondrial membrane permeabilization.²

Recent data suggests that Cyt. C release follows a biphasic kinetics rather than in an all-or-nothing manner as previously believed. An in vitro study identifies Cyt. C as a messenger that coordinates mitochondrial-endoplasmic reticulum interactions that drive apoptosis.³⁰ Early in apoptosis, Cyt. C translocates to the endoplasmic reticulum where it selectively binds to inositol 1,4,5-triphosphate receptor (InsP(3)R), resulting in sustained, oscillatory cytosolic Ca^{2+} increases.³⁰ These Ca^{2+} signaling events are linked to the coordinate release of Cyt. C from all mitochondria. These findings identify a feed-forward mechanism whereby early Cyt. C release increases InsP(3)R function, resulting in augmented Cyt. C release then amplifies the apoptotic signal.^{30,31} Cyt. C also interacts with InsP(3)R type 1 and ryanodine receptor type 2 in gerbil hippocampus subjected to transient brain ischemia with a short reperfusion, and contributes to postischemic neuronal death.³² These findings illustrate the importance of intracellular Ca^{2+} stores in apoptosis, and ischemic cell damage.

Secondary Active Transport Proteins and Ca^{2+} Overload

Loss of Ca^{2+} homeostasis in the cell plays a critical role in pathogenesis of ischemic mitochondrial dysfunction and subsequent cell damage. Recent studies show that the important secondary active transport proteins, NKCC and NCX, contribute to ischemia-induced dissipation of ion homeostasis.

NKCC. The electroneutral NKCC protein belongs to the cation-chloride cotransporter family, which transports Na^+ , K^+ and Cl^- into cells under physiological conditions with a stoichiometry of $1\text{Na}^+:1\text{K}^+:2\text{Cl}^-$.^{33,34} The transmembrane chemical gradients for Na^+ , K^+ and Cl^- determine net inward ion movement via NKCC.³⁴ In most cells, the driving force for ion influx is in part supplied by the inward Na^+ gradient, maintained by Na^+/K^+ -ATPase. However, in Cl^- secretory epithelial cells, the Cl^- gradient is also an important driving force for ion influx.³⁴ NKCC is characteristically inhibited by the sulfamoybenzoic acid loop diuretics, such as bumetanide and furosemide.³⁵ 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.^{33,36}

NKCC serves multiple functions, including ion and fluid movements in secreting or reabsorbing epithelia and cell volume regulation.^{33,34} However, understanding of the role of NKCC1 in the central nervous system just began. NKCC1 is important in

the maintenance of intracellular Cl^- in neurons and contributes to GABA-mediated depolarization in immature neurons. Thus, NKCC1 may affect neuronal excitability through regulation of intracellular Cl^- concentration.^{37,38} NKCC1 may also contribute to K^+ clearance and maintenance of intracellular Na^+ in astrocytes and oligodendrocytes. Recent studies suggest that high $[\text{K}^+]_o$ activation of NKCC1 leads to astrocyte swelling and glutamate release, as well as neuronal Na^+ , and Cl^- influx during acute excitotoxicity.³⁹ In addition, inhibition of NKCC1 activity significantly reduces infarct volume and cerebral edema following cerebral focal ischemia.³⁹

NCX. NCX belongs to the Ca^{2+} /cation antiporter superfamily. It is a ubiquitous transporter that plays an important role in regulating and maintaining cellular Ca^{2+} balance in various tissues. At present, three isoforms in the NCX family have been identified: NCX1, NCX2 and NCX3.⁴⁰ They share biophysical and biochemical properties but exhibit differences in expression during development and in various adult tissues. NCX1 is present in most tissues and is highly expressed in heart, brain and kidney, while NCX2 and NCX3 are expressed mainly in the brain and skeletal muscles.⁴⁰⁻⁴² Immunogold EM studies reveal that neuronal NCXs are preferentially located in dendrites and dendritic spines, while glial NCXs are prominently expressed in astrocytic processes ensheathing excitatory synapses.⁴³ The specific localization indicates that NCXs are favourably located for buffering $\text{Ca}^{2+}_{\text{cyt}}$ in excitatory postsynaptic sites and may participate in shaping astrocytic $\text{Ca}^{2+}_{\text{cyt}}$ transients evoked by ongoing synaptic activity.⁴³

NCX works bidirectionally depending on cytosolic Na^+ concentration ($[\text{Na}^+]_{\text{cyt}}$), cytosolic Ca^{2+} concentration ($[\text{Ca}^{2+}]_{\text{cyt}}$) and plasma membrane potential. The stoichiometry of NCX1 is generally accepted to be 3 Na^+ ions/1 Ca^{2+} ion. Thus, NCX1 transport is electrogenic, and dependent on plasma membrane potential.⁴⁴ Under physiological conditions, 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 conditions in which Na^+ accumulates inside the cell such as during ischemia, NCX may work in reverse mode-operation and conduct Ca^{2+} influx.

Roles of NKCC1 and NCX in ischemic mitochondrial dysfunction. Loss of Na^+ homeostasis in cells following in vitro hypoxia/ischemia. A steep inwardly directed Na^+ gradient is essential for cell functions, such as glutamate reuptake and regulation of intracellular ion concentrations by other secondary ion transporters.^{46,47} Dissipation of the Na^+ gradient is one of the key elements in promoting cellular damage in cells during energy failure.^{47,48}

A recent study shows that one hour oxygen-glucose deprivation (OGD) triggers an approximately four-fold increase in $[\text{Na}^+]_{\text{cyt}}$ in cultured cortical neurons.¹¹ Additionally, 60 min reoxygenation (REOX) following two hour OGD can induce a seven-fold increase in $[\text{Na}^+]_{\text{cyt}}$ in neurons.⁴⁸ An ~3.6-fold increase in $[\text{Na}^+]_{\text{cyt}}$ after OGD is also reported in cultured astrocytes.⁷ Additionally, Kintner DB, et al. reported that the onset of REOX following either of two hypoxic conditions, OGD and HAIR (a hypoxic, acidic, ion-shifted ringer buffer), triggers a rapid Na^+_{cyt} overload in astrocytes.⁸ This finding is consistent with reports on $[\text{Na}^+]_{\text{cyt}}$ accumulation in rat spinal cord astrocytes,⁴⁹ rat cortical astrocytes⁴⁷ and mouse cortical astrocytes,⁵⁰ when these cells are exposed to glucose deprivation, NaN_3 -mediated chemical hypoxia and simulated ischemia, respectively.

Lenart B, et al. reported that NKCC1 activity in cortical astrocytes is increased during reoxygenation (REOX) via protein

phosphorylation, which is a major regulatory mechanism for stimulation of NKCC1 activity.^{7,51} It also has been reported that loss of NKCC1 activity either by treatment with its inhibitor bumetanide or by genetic ablation blocks ~64% of the rise in Na^+_{cyt} in astrocytes,⁸ and results in ~50% less Na^+_{cyt} accumulation in neurons.¹¹ The remaining Na^+_{cyt} accumulation could be the result of other Na^+ influx pathways (such as voltage-gated Na^+ channels, Na^+/H^+ exchanger, etc.) or a decrease in Na^+ extrusion via Na^+/K^+ ATPase during ischemia.^{7,11} In addition, NKCC1 also plays a role during glutamate-mediated excitotoxicity. $[\text{Na}^+]_{\text{cyt}}$ accumulation is found in cultured oligodendrocytes exposed to alphaamino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) and cyclothiazide (CTZ),¹⁰ and in cortical neurons exposure to 60 μM N-methyl-D-aspartic acid (NMDA).³⁸ Blocking NKCC1 activity with bumetanide significantly reduces the AMPA/CTZ-induced Na^+ influx in oligodendrocytes¹⁰ as well as the accumulation of Na^+_{cyt} content after NMDA receptor activation in neurons.³⁸ Thus, these results imply that stimulation of NKCC1 activity evokes excessive Na^+ entry and disrupts Na^+ homeostasis following hypoxia/ischemia.

Intracellular Na^+ overload leads to reversal of NCX. Reduction in the Na^+ gradient affects Ca^{2+} homeostasis in cells. Ca^{2+} entry pathways involve L-type voltage-gated Ca^{2+} channels, ionotropic glutamate receptors, and NCX_{rev} .⁵² Li et al. reported that elevation of $[\text{Na}^+]_{\text{cyt}}$ increases $\text{Ca}^{2+}_{\text{cyt}}$ via NCX_{rev} in neurons and causes irreversible injury during anoxia and ischemia.⁵³ An increase in $\text{Ca}^{2+}_{\text{cyt}}$ via NCX_{rev} occurs in rat astrocytes when $[\text{Na}^+]_{\text{cyt}}$ is raised by inhibition of Na^+/K^+ -ATPase activity or activation of AMPA channels.^{54,55} Furthermore, Ca^{2+} entry is detected in astrocytes after OGD. Inhibition of NCX_{rev} with 3 μM KB-R7943 abolishes the OGD-mediated increase in releasable Ca^{2+} in bradykinin-sensitive Ca^{2+} stores.⁷ These findings further suggest that NCX_{rev} plays a role in Ca^{2+} entry and Ca^{2+} signaling in cells after OGD. So far, more benzyloxyphenyl derivatives (SEA0400, SN-6 and YM-244769) have been developed as selective NCX_{rev} inhibitors.⁴⁵ They effectively prevent ischemia-reperfusion injury in several animal models.⁴⁵ Thus, NCX inhibitors may have therapeutic potential as novel drugs for reperfusion injury.

In addition to a significant rise in $[\text{Ca}^{2+}]_{\text{cyt}}$ during HAIR, REOX induces a secondary increase in $[\text{Ca}^{2+}]_{\text{cyt}}$.⁸ Approximately 70% of the REOX-triggered Ca^{2+} rise following 15 min HAIR is abolished with 10 μM KB-R7943. Interestingly, NKCC1^{-/-} astrocytes exhibit a much lower level of REOX-triggered Ca^{2+} rise.⁸ The data suggest that Ca^{2+} entry during REOX is largely via NKCC1-mediated reversal of NCX in astrocytes. This finding is consistent with thermodynamic modeling that predicts NCX will operate in the reverse mode and mediate Ca^{2+} influx when $[\text{Na}^+]_{\text{cyt}}$ in astrocytes is increased ~25 mM.⁵⁶ Maximum values of $[\text{Na}^+]_{\text{cyt}}$ reached 70–80 mM during REOX following either OGD or HAIR, a condition that favors NCX_{rev} . This view is supported by several recent reports. For example, inhibition of NCX with 100 nM KB-R7943 significantly attenuates the rise in $[\text{Ca}^{2+}]_{\text{cyt}}$ in response to severe mechanical strain injuries in rat cortical astrocytes. The strain injury leads to a rapid rise in $[\text{Na}^+]_{\text{cyt}}$ in astrocytes that is sustained for ~50 min.⁵⁷

Moreover, a transient elevation of $[\text{Ca}^{2+}]_{\text{cyt}}$ following 25–30 min HAIR is blocked by NCX_{rev} inhibitor KB-R7943.⁵⁸ The effects of KB-R7943 imply that REOX evokes Na^+ entry, which subsequently triggers the reversal of NCX. The elevation of $[\text{Na}^+]_{\text{cyt}}$ and the

reversal of NCX have been suggested to play a role in spinal cord astrocyte ischemic damage during the reperfusion period.⁴⁹ NCX inhibitors bepridil and KB-R7943 improve functional recovery of white matter tracts after anoxic and traumatic injury.⁵³ In addition, Ca^{2+} influx via NCX_{rev} is decreased by approximately 70% in $\text{NCX}^{+/-}$ neurons, which exhibit reduced NCX1 protein expression compared to $\text{NCX}^{+/+}$ neurons.⁵⁹ KB-R7943 treatment attenuates the transient Ca^{2+} influx by 60% and sustained Ca^{2+} influx by 70% in oligodendrocytes.¹⁰ Moreover, inhibition of NKCC1 activity with bumetanide reduced the sustained Ca^{2+} rise by 50%.¹⁰ These data suggest that NKCC1 and NCX_{rev} are involved in AMPA-triggered Ca^{2+} signaling in oligodendrocytes. The protective effects, via inhibition of NKCC1 and NCX_{rev} on $\text{Ca}^{2+}_{\text{cyt}}$ overload, may result from reducing $\text{Na}^{+}_{\text{cyt}}$ overload and inhibition of NCX_{rev} .¹⁰

$\text{Ca}^{2+}_{\text{m}}$ overload, mitochondrial dysfunction and cell death. The mechanisms of ischemic injury include Ca^{2+} entry and Ca^{2+} -mediated cell death. $\text{Ca}^{2+}_{\text{m}}$ overload leads to mitochondrial dysfunction via many pathways, including inhibition of oxidative phosphorylation, oxygen free radical formation or formation of the mitochondrial PTP, which subsequently releases proapoptotic molecules such as Cyt. C.⁵ OGD/REOX can cause a dramatic increase in $\text{Ca}^{2+}_{\text{m}}$ accumulation and Cyt. C release in cultured neurons.⁴⁸ An approximately five-fold increase of $\text{Ca}^{2+}_{\text{m}}$ accompanies an ~58% depolarization of Ψ_{m} and subsequent Cyt. C release in cortical astrocytes at 1 h REOX following 2 h OGD (Figs. 1 and 2).⁸ In addition, REOX following 15 min HAIR can cause significant dissipation of Ψ_{m} and $\text{Ca}^{2+}_{\text{m}}$ increase in $\text{NKCC1}^{+/+}$ astrocytes. Pharmacological inhibition or genetic ablation of NKCC1 not only attenuates REOX-induced $\text{Ca}^{2+}_{\text{m}}$ overload and Ψ_{m} depolarization, but it also blocks Cyt. C release from mitochondria and results in less cell death in culture astrocytes (Figs. 1 and 2).⁸ Moreover, in $\text{NCX1}^{+/-}/\text{NKCC1}^{+/-}$ double heterozygous neurons, with lower NCX1 and NKCC1 protein expression, NCX-mediated Ca^{2+} influx is nearly abolished. These neurons exhibit approximately 65% less neuronal death and increased tolerance to ischemic damage.⁹ Studies in glutamate-mediated excitotoxicity in oligodendrocyte damage shows the AMPA/CTZ-elicited $\text{Ca}^{2+}_{\text{cyt}}$ increase, $\text{Ca}^{2+}_{\text{m}}$ overload, mitochondrial damage, and cell death were significantly reduced by inhibiting NKCC1 or NCX_{rev} .¹⁰ In summary, these findings suggest that NKCC1 in conjunction with NCX1 mediate dysregulation of cellular Na^{+} and Ca^{2+} homeostasis and can seriously impair cell function and survival.⁸

Conclusion

Loss of ionic homeostasis plays an important role in ischemic brain damage. Recent findings suggest that NKCC and NCX_{rev} are involved in dissipation of ion homeostasis and mitochondrial dysfunction. The concerted activities of multiple ion transport proteins not only perturb intracellular Na^{+} and Ca^{2+} homeostasis in response to hypoxia/ischemia, but also affect mitochondrial Ca^{2+} and Cyt. C release. Therefore, these ion transport proteins may be potential therapeutic targets to reduce or prevent ischemia-mediated loss of ion homeostasis.

Acknowledgements

We thank Douglas Kintner for critical reading of the manuscript and many helpful suggestions. This work was supported by National Institutes of Health Grant R01NS-38118 (D.S.),

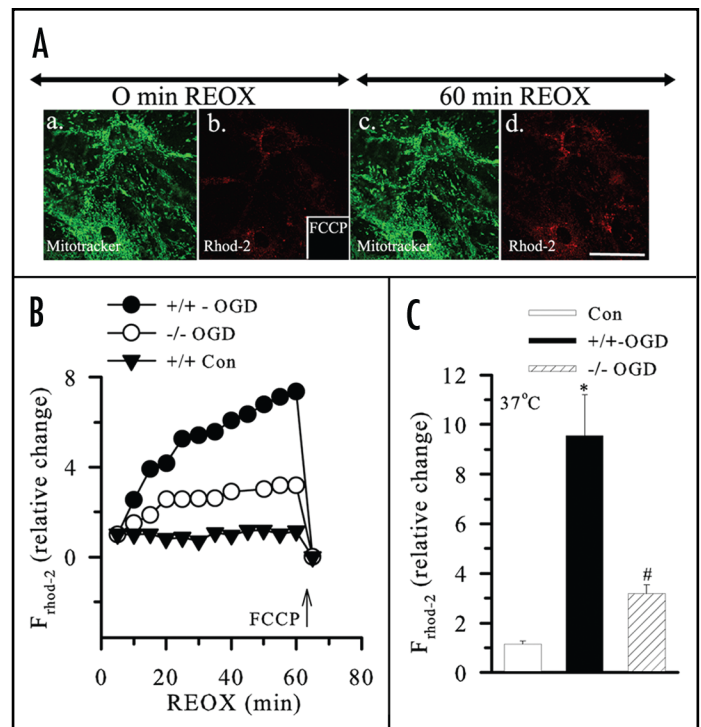


Figure 1. Changes in mitochondrial Ca^{2+} ($\text{Ca}^{2+}_{\text{m}}$) in astrocytes following OGD/REOX. (A) Mitotracker (green in a) and Rhod-2 (red in b) images were shown in $\text{NKCC1}^{+/+}$ astrocytes at the end of 2 h OGD and at 60 min REOX (c and d). Scale bar = 12.3 μm . (B) Average tracings of relative change in Rhod-2 fluorescence during 60 min REOX following 2 h OGD in $\text{NKCC1}^{+/+}$ and $\text{NKCC1}^{-/-}$ astrocytes. Arrow: response of Rhod-2 fluorescence to FCCP. (C) Summary data. Data are means \pm SEM; $n = 4-5$. * $p < 0.05$ vs. control; # $p < 0.05$ vs. $\text{NKCC1}^{+/+}$ OGD/REOX. Reprinted with permission from Kinter et al.⁸

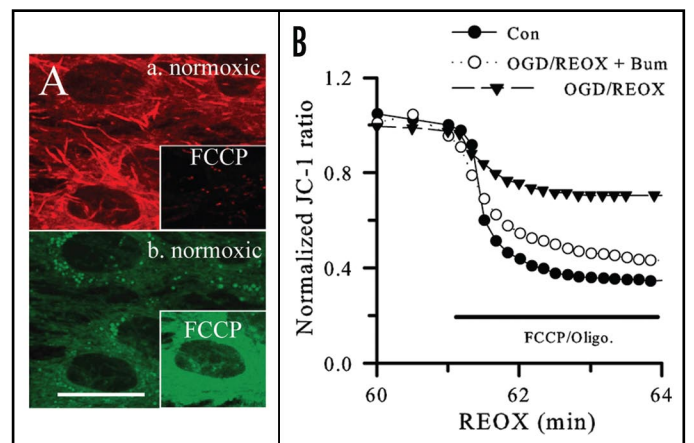


Figure 2. Changes in membrane potential (Ψ_{m}) in astrocytes following OGD/REOX. (A) JC-1 aggregate (a) and monomer (b) fluorescence in $\text{NKCC1}^{+/+}$ cells under normoxic control conditions. Insets in (A): changes of JC-1 fluorescence following 3 min of FCCP (10 μM). Scale bar = 12.3 μm . (B) A representative tracing of changes of the JC-1 ratios. $n = 4-5$. Reprinted with permission from Kinter et al.⁸

Tsinghua-Yue-Yuen Medical Sciences Fund (X.J.L), and China Scholarship Council Postgraduate Scholarship Program (Y.L).

References

- McBride HM, Neuspiel M, Wasiak S. Mitochondria: more than just a powerhouse. *Curr Biol* 2006; 16:551-60.
- Orrenius S, Zhivotovsky B, Nicotera P. Regulation of cell death: the calcium-apoptosis link. *Nat Rev Mol Cell Biol* 2003; 4:552-65.
- Bernardi P and Rasola A. Calcium and cell death: the mitochondrial connection. *Subcell Biochem* 2007; 45:481-506.
- Hoyt KR, Stout AK, Cardman JM, Reynolds IJ. The role of intracellular Na⁺ and mitochondria in buffering of kainate-induced intracellular free Ca²⁺ changes in rat forebrain neurones. *J Physiol (Lond)* 1998; 509:103-16.
- Brookes PS, Yoon Y, Robotham JL, Anders MW, Sheu SS. Calcium, ATP and ROS: a mitochondrial love-hate triangle. *Am J Physiol Cell Physiol* 2004; 287:R17-33.
- Cao G, Xiao M, Sun F, Xiao X, Pei W, Li J, et al. Cloning of a novel Apaf-1-interacting protein: a potent suppressor of apoptosis and ischemic neuronal cell death. *J Neurosci* 2004; 24:6189-201.
- Lenart B, Kintner DB, Shull GE, Sun D. Na-K-Cl cotransporter-mediated intracellular Na⁺ accumulation affects Ca²⁺ signaling in astrocytes in an in vitro ischemic model. *J Neurosci* 2004; 24:9585-97.
- Kintner DB, Luo J, Gerdts J, Ballard AJ, Shull GE, Sun D. Role of Na⁺-K⁺-Cl⁻ cotransport and Na⁺/Ca²⁺ exchange in mitochondrial dysfunction in astrocytes following in vitro ischemia. *Am J Physiol Cell Physiol* 2007; 292:1113-22.
- Luo J, Wang Y, Chen H, Kintner DB, Cramer SW, Gerdts JK, et al. A concerted role of Na(+)-K(+)-Cl(-) cotransporter and Na(+)/Ca(2+) exchanger in ischemic damage. *J Cereb Blood Flow Metab* 2008; 28:737-46.
- Chen H, Kintner DB, Jones M, Matsuda T, Baba A, Kiedrowski L, et al. AMPA-mediated excitotoxicity in oligodendrocytes: role for Na(+)-K(+)-Cl(-) co-transport and reversal of Na(+)/Ca(2+) exchanger. *J Neurochem* 2007; PM:17490438.
- Chen H, Luo J, Kintner DB, Shull GE, Sun D. Na⁺-dependent chloride transporter (NKCC1)-null mice exhibit less gray and white matter damage after focal cerebral ischemia. *J Cereb Blood Flow Metab* 2005; 25:54-66.
- Clapham DE. Calcium signaling. *Cell* 1995; 80:259-68.
- Fall CP and Keizer JE. Mitochondrial modulation of intracellular Ca(2+) signaling. *J Theor Biol* 2001; 210:151-65.
- Lipton P. Ischemic cell death in brain neurons. *Physiol Rev* 1999; 79:1431-568.
- Saris NE and Eriksson KO. Mitochondrial dysfunction in ischaemia-reperfusion. *Acta Anaesthesiol Scand Suppl* 1995; 107:171-6.
- Grinwald PM. Calcium uptake during post-ischemic reperfusion in the isolated rat heart: influence of extracellular sodium. *J Mol Cell Cardiol* 1982; 14:359-65.
- Gunter TE, Yule DI, Gunter KK, Eliseev RA, Salter JD. Calcium and mitochondria. *FEBS Lett* 2004; 567:96-102.
- Crompton M. The mitochondrial permeability transition pore and its role in cell death. *Biochem J* 1999; 341:233-49.
- Brustovetsky N, Brustovetsky T, Jemmerson R, Dubinsky JM. Calcium-induced cytochrome c release from CNS mitochondria is associated with the permeability transition and rupture of the outer membrane. *J Neurochem* 2002; 80:207-18.
- Hunter DR and Haworth RA. The Ca²⁺-induced membrane transition in mitochondria I. The protective mechanisms. *Arch Biochem Biophys* 1979; 195:453-9.
- Haworth RA and Hunter DR. The Ca²⁺-induced membrane transition in mitochondria II. Nature of the Ca²⁺ trigger site. *Arch Biochem Biophys* 1979; 195:460-7.
- Rasola A and Bernardi P. The mitochondrial permeability transition pore and its involvement in cell death and in disease pathogenesis. *Apoptosis* 2007; 12: 815-833.
- Strasser A, O'Connor L, Dixit VM. Apoptosis signaling. *Annu Rev Biochem* 2000; 69:217-45.
- Eskes R, Desagher S, Antonsson B, Martinou JC. Bid induces the oligomerization and insertion of Bax into the outer mitochondrial membrane. *Mol Cell Biol* 2000; 20:929-35.
- Antonsson B, Conti F, Ciavatta A, Montessuit S, Lewis S, Martinou I, et al. Inhibition of Bax channel-forming activity by Bcl-2. *Science* 1997; 277:370-2.
- Lindsten T, Ross AJ, King A, Zong WX, Rathmell JC, Shiels HA, et al. The combined functions of proapoptotic Bcl-2 family members bak and bax are essential for normal development of multiple tissues. *Mol Cell* 2000; 6:1389-99.
- Ott M, Robertson JD, Gogvadze V, Zhivotovsky B, Orrenius S. Cytochrome c release from mitochondria proceeds by a two-step process. *Proc Natl Acad Sci USA* 2002; 99:1259-63.
- Petrosillo G, Ruggiero FM, Pistolese M, Paradies G. Reactive oxygen species generated from the mitochondrial electron transport chain induce cytochrome c dissociation from beef-heart submitochondrial particles via cardiolipin peroxidation. Possible role in the apoptosis. *FEBS Lett* 2001; 509:435-8.
- Grijalba MT, Veccesi AE, Schreier S. Ca²⁺-induced increased lipid packing and domain formation in submitochondrial particles. A possible early step in the mechanism of Ca²⁺-stimulated generation of reactive oxygen species by the respiratory chain. *Biochemistry* 1999; 38:13279-87.
- Boehning D, Patterson RL, Sedaghat L, Glebova NO, Kurosaki T, Snyder SH. Cytochrome c binds to inositol (1,4,5) trisphosphate receptors, amplifying calcium-dependent apoptosis. *Nat Cell Biol* 2003; 5:1051-61.
- Boehning D, Patterson RL, Snyder SH. Apoptosis and calcium: new roles for cytochrome c and inositol 1,4,5-trisphosphate. *Cell Cycle* 2004; 3:252-4.
- Beresewicz M, Kowalczyk JE, Zablocka B. Cytochrome c binds to inositol (1,4,5) trisphosphate and ryanodine receptors in vivo after transient brain ischemia in gerbils. *Neurochem Int* 2006; 48:568-71.
- Haas M and Forbush B, III. The Na-K-Cl cotransporter of secretory epithelia. *Annu Rev Physiol* 2000; 62:515-34.
- Russell JM. Sodium-potassium-chloride cotransport. *Physiol Rev* 2000; 80:211-76.
- Forbush B, III and Palfrey HC. [³H]bumetanide binding to membranes isolated from dog kidney outer medulla. Relationship to the Na, K, Cl co-transport system. *J Biol Chem* 1983; 258:11787-92.
- Xu JC, Lytle C, Zhu TT, Payne JA, Benz E Jr, Forbush B, III. Molecular cloning and functional expression of the bumetanide-sensitive Na-K-Cl cotransporter. *Proc Natl Acad Sci USA* 1994; 91:2201-5.
- Schomberg SL, Su G, Haworth RA, Sun D. Stimulation of Na-K-2Cl cotransporter in neurons by activation of Non-NMDA ionotropic receptor and group-I mGluRs. *J Neurophysiol* 2001; 85:2563-75.
- Beck J, Lenart B, Kintner DB, Sun D. Na-K-Cl cotransporter contributes to glutamate-mediated excitotoxicity. *J Neurosci* 2003; 23:5061-8.
- Su G, Kintner DB, Sun D. Contribution of Na(+)-K(+)-Cl(-) cotransporter to high-[K(+)] (o)-induced swelling and EAA release in astrocytes. *Am J Physiol Cell Physiol* 2002; 282:1136-46.
- Annunziato L, Pignataro G, Di Renzo GF. Pharmacology of brain Na(+)/Ca(2+) exchanger: from molecular biology to therapeutic perspectives. *Pharmacol Rev* 2004; 56:633-54.
- Lee SL, Yu AS, Lytton J. Tissue-specific expression of Na(+)-Ca(2+) exchanger isoforms. *J Biol Chem* 1994; 269:14849-52.
- Nicoll DA, Ottolia M, Philipson KD. Toward a topological model of the NCX1 exchanger. *Ann N Y Acad Sci* 2002; 976:11-8.
- Minelli A, Castaldo P, Gobbi P, Salucci S, Magi S, Amoroso S. Cellular and subcellular localization of Na(+)-Ca(2+) exchanger protein isoforms, NCX1, NCX2 and NCX3 in cerebral cortex and hippocampus of adult rat. *Cell Calcium* 2007; 41:221-34.
- Kang TM and Hilgemann DW. Multiple transport modes of the cardiac Na⁺/Ca²⁺ exchanger. *Nature* 2004; 427:544-8.
- Iwamoto T. Na(+)/Ca(2+) exchange as a drug target--insights from molecular pharmacology and genetic engineering. *Ann N Y Acad Sci* 2007; 1099:516-28.
- Walz W. Role of glial cells in the regulation of the brain ion microenvironment. *Prog Neurobiol* 1989; 33:309-33.
- Longuemare MC, Rose CR, Farrell K, Ransom BR, Waxman SG, Swanson RA. K⁺-induced reversal of astrocyte glutamate uptake is limited by compensatory changes in intracellular Na⁺. *Neuroscience* 1999; 93:285-92.
- Luo J, Chen H, Kintner DB, Shull GE, Sun D. Decreased neuronal death in Na⁺/H⁺ exchanger isoform 1-null mice after in vitro and in vivo ischemia. *J Neurosci* 2005; 25:11256-68.
- Rose CR, Waxman SG, Ransom BR. Effects of glucose deprivation, chemical hypoxia and simulated ischemia on Na⁺ homeostasis in rat spinal cord astrocytes. *J Neurosci* 1998; 18:3554-62.
- Silver IA, Deas J, Erecinska M. Ion homeostasis in brain cells: differences in intracellular ion responses to energy limitation between cultured neurons and glial cells. *Neuroscience* 1997; 78:589-601.
- Flemmer AW, Gimenez I, Dowd BF, Darman RB, Forbush B. Activation of the Na-K-Cl cotransporter NKCC1 detected with a phospho-specific antibody. *J Biol Chem* 2002; 277:37551-8.
- Stys PK. Anoxic and ischemic injury of myelinated axons in CNS white matter: from mechanistic concepts to therapeutics. *J Cereb Blood Flow Metab* 1998; 18:2-25.
- Li S, Jiang Q, Stys PK. Important role of reverse Na⁺-Ca²⁺ exchange in spinal cord white matter injury at physiological temperature. *J Neurophysiol* 2000; 84:1116-9.
- Goldman WF, Yarowsky PJ, Juhaszova M, Krueger BK, Blaustein MP. Sodium/calcium exchange in rat cortical astrocytes. *J Neurosci* 1994; 14:5834-43.
- Smith JP, Cunningham LA, Partridge LD. Coupling of AMPA receptors with the Na⁺/Ca²⁺ exchanger in cultured rat astrocytes. *Brain Res* 2000; 887:98-109.
- Kintner DB, Look A, Shull GE, Sun D. 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* 2005; 289:934-45.
- Floyd CL, Gorin FA, Lyeth BG. Mechanical strain injury increases intracellular sodium and reverses Na⁺/Ca²⁺ exchange in cortical astrocytes. *Glia* 2005; 51:35-46.
- Bondarenko A, Svichar N, Chesler M. Role of Na⁺-H⁺ and Na⁺-Ca²⁺ exchange in hypoxia-related acute astrocyte death. *Glia* 2005; 49:143-52.
- Luo J, Wang Y, Chen H, Kintner DB, Cramer SW, Gerdts JK, et al. A concerted role of Na(+)-K(+)-Cl(-) cotransporter and Na(+)/Ca(2+) exchanger in ischemic damage. *J Cereb Blood Flow Metab* 2008; 28, 737-746.