Ion Transport Systems as Targets of Free Radicals During Ischemia Reperfusion Injury

J. Lehotský[1], P. Kaplán[1], M. Matejovičová[1], R. Murín[1], P. Račay[1], and L. Raeymaekers[2]

1 *Department of Medical Biochemistry, Jessenius Medical Faculty,*
  Comenius University, Martin, Slovakia
2 *Laboratory for Physiology, Catholic University Leuven,*
  B–3000 Leuven, Belgium

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Abstract. Oxidative stress is a recognized pathogenic factor in ischemia/reperfusion injury (IRI). Iron induced generation of reactive oxygen species (ROS) *in vitro* reduces both the Na^+,K^+-ATPase activity and Na^+-Ca^{2+} exchanger of synaptosomal membranes, concomitantly with alteration of physical state of membranes. Oxidative insult also leads to the loss of ability of endoplasmic reticular membranes (ER) to sequester Ca^{2+} as well as to the increase of Ca^{2+} permeability. Furthermore, ROS induces both lipid peroxidation and lipid-independent modifications of membrane proteins. Acute *in vivo* ischemia alters kinetic parameters of Na^+,K^+-ATPase affecting mainly the dephosphorylation step of ATPase cycle with parallel changes of Na^+-Ca^{2+} exchanger and alterations of physical membrane environment. Subsequent reperfusion after ischemia is associated with decrease of immuno signal for PMCA 1 isoform in hippocampus. In addition, incubation of non-ischemic membranes with cytosol from ischemic hippocampus decreases level of PMCA 1 in non-ischemic tissues. Loss of PMCA 1 protein is partially protected both by calpain- and by non-specific protease inhibitors which suggest possible activation of proteases in the reperfusion period. On the other hand, ischemia does not affect the level of Ca^{2+} pump (SERCA 2b) and calreticulin of intracellular Ca^{2+} stores. However, IRI resulted in a decrease of IP$_3$ receptor I and altered active Ca^{2+} accumulation into the ER. A non-specific alteration of physical properties of total membranes such as the oxidative modifications of proteins as well as the content of lipoperoxidation products can also be detected after IRI. ROS can alter physical and functional properties of neuronal membranes. We discuss our results suggesting

Correspondence to: Prof. Ján Lehotský, Ph.D., Department of Medical Biochemistry, Jessenius Medical Faculty, Comenius University, Malá Hora 4, 036 01 Martin, Slovakia
E-mail: Lehotsky@jfmed.uniba.sk

that ischemia-induced disturbance of ion transport systems may participate in or follow delayed death of neurons after ischemia.

Introduction

Current evidence suggests that loss of ion homeostasis is involved in the common pathways leading to cell death. Calcium is a ubiquitous second messenger controlling many physiological functions of neuronal and glial cells. Several 'passive' and 'active' mechanisms are responsible for maintenance of the low intracellular Ca^{2+} level and for its restoration after transient physiological elevation such as Ca^{2+} channels, Ca^{2+} binding proteins, Ca^{2+} pumps in the plasma membrane (PMCA) and in the internal Ca^{2+} stores (SERCA). In addition, Na^+-Ca^{2+} exchanger activity linked with the function of the Na^+,K^+-ATPase mutually maintains free Ca^{2+} and Na^+ concentrations at physiological levels. In neural cells, the relative contribution of these components to the overall ion homeostasis varies (Kostyuk and Verkhratsky 1994, Račay et al. 1996).

Neuronal ischemia/reperfusion injury (IRI) is believed to be linked with intra-cellular disregulation of ion homeostasis including Ca^{2+} homeostasis, which leads to triggering of ion Ca^{2+}-dependent bio-polymer degradation, concomitant generation of free radicals, mitochondrial and bioenergetic failure, and ultimately to the activation of reactions culminating to necrotic/apoptotic cell death. However, it is not yet clear which sources of Ca^{2+} and which pathways are involved (Kristian and Siesjö 1998). Oxidative stress, initiated mainly by reactive oxygen/nitrogen species [RO(N)S] is recognized as a pathogenic factor in IRI. The brain is especially vulnerable to free radical damage. The disruptive action of RO(N)S involves lipid peroxidation, protein modifications and DNA oxidation. Each of these events may cause alterations of the membrane structure and function, including membrane fluidity, permeability, activity of enzymes, channels, transport proteins and receptors (Lipton 1999). The changes of redox balance also lead to activation or silencing of many susceptible genes (Dalton et al. 1999).

Plasma membrane ion transport systems as targets of ischemia reperfusion damage including reactive oxygen species

The Na^+,K^+-ATPase, the enzyme that maintains Na^+, and K^+ gradients across the plasma membrane was reported to be inhibited by ROS in the brain. The results suggest that effect of ROS on this enzyme may be very specific and may include selective alterations of its active sites. The Na^+-Ca^{2+} exchanger is a calcium transport system of low affinity and high capacity and its activity is closely coupled with the activity of Na^+, K^+-ATPase. Effect of ROS on this system in the brain is not well-known yet (Lipton 1999). In our experiments we showed that iron generated ROS *in vitro* reduced both the Na^+, K^+-ATPase activity and the activity of Na^+-Ca^{2+} exchanger of synaptosomal membranes. Physical state of the membrane is also altered as can be seen from reduction of membrane fluidity analyzed by fluorescence anisotropy measurement. Likewise, an acute *in vivo* ischemic

insult alters kinetic parameters of Na^+,K^+-ATPase, affecting mainly the dephosphorylation step of ATPase cycle with parallel changes of Na^+-Ca^{2+} exchanger and alterations of physical membrane environment (Matejovičová et al. 1996).

The PMCA pump is a ubiquitously expressed protein with high affinity for Ca^{2+}. Together with the Na^+-Ca^{2+} exchanger it is responsible for the extrusion of Ca^{2+} from the cytosol (Lehotsky 1995). In humans and rats, the alternatively spliced variants of four isoforms of PMCA protein are transcribed in neurons and glia cells (Fresu et al. 1999; Garcia and Strehler 1999). Recent *in vitro* studies performed on erythrocytes, smooth muscle, heart and brain (Rohn et al. 1993; McConnell et al. 1999; Zaidi and Michaelis 1999) as well as *in vivo* experiments on neuronal hypoxia/ischemia (Lipton 1999) have documented ROS-induced alterations of plasma membrane integrity, cross-linking and aggregation of PMCA molecules in parallel with inhibition of the catalytic activity of PMCA pump. In Mongolian gerbil, similarly to other rodents, we detected isoforms of the PMCA protein in brain, however, with a distinct distribution pattern (Lehotsky et al. 1999a). Transient ischemia did not affect the level of any PMCA isoforms. However, ischemia followed by reperfusion up to 7 to 10 days led to a remarkable signal decrease localized in the hippocampus, which is thought to be the most affected brain region (CA1 sector). The decrease could be ascribed solely to the loss of PMCA1 since other isoforms had shown no significant differences neither in the hippocampus nor in the cortex. In addition, incubation of non-ischemic membranes with cytosol from ischemic hippocampus decreased level of PMCA 1 in non-ischemic tissues. Loss of protein level is partially protected both by calpain- and non-specific protease inhibitors which suggests possible activation of calpains and/or caspases-like proteases in the reperfusion period. Cerebral ischemia is known to alter proteosynthetic and proteolytic machinery (Paschen and Doutheil 1999). Moreover, an increased protein hydrophobicity as a result of alteration induced by oxidants is a signal for higher susceptibility to degradation by several proteases (Pacifici et al. 1993). A massive disappearance of PMCA activity and alteration of PMCA1 as well as PMCA2 at the mRNA and protein levels has been associated with neuronal death induced either by ischemia (Oguro et al. 1995) or kainic acid induced seizure (Garcia et al. 1997). Moreover, in neurons, fluctuation of $[Ca^{2+}]_i$ alone mediates changes in the expression of alternative spliced PMCA variants (Garcia and Strehler 1999). Although no literature data is available on the neural regulation of PMCA isoforms on gene and mRNA level or controlled isoform proteolysis and we have no answer yet at which level the changes occur, it seems that PMCA is an important candidate in Ca^{2+} mediated delayed neuronal death.

Intracellular calcium store transport systems as targets of ischemia reperfusion damage including reactive oxygen species

Endoplasmic reticulum (ER) plays a co-ordinating role in intracellular Ca^{2+} signalling in neurones and glia. Alteration in the equilibrium of endoplasmic Ca^{2+} sequestration/release changes multiple signalling routes and strongly affects neuronal functioning and survival (Kostyuk and Verkhratsky 1994; Yao et al. 1999).

The ER membranes contain both SERCA type Ca^{2+} pump isoforms (SERCA 2b and SERCA 3) and Ca^{2+} channels that mediate the uptake and release of Ca^{2+} into/from the store. Depletion of ER Ca^{2+} pools activates a stress response (suppression of global proteosynthesis and activation of stress genes) and it is thought that disturbances of the ER functions may be involved in stress (ischemia, seizure)-induced cell injury in which stress response protein serves a neuroprotective action (Paschen and Doutheil 1999).

Similarly to other tissues, the alkoxyl and peroxyl radicals generated by iron or hydroxyl radicals generated by Fenton reaction lead to the loss of ability of neuronal ER membranes to sequester Ca^{2+}, as well as to the increase of Ca^{2+} permeability and to the decrease of Ca^{2+}-ATPase activity. The potency of two ROS generated systems to decrease membrane fluidity correlated well with the system's potency to induce lipoperoxidation (Lehotsky et al. 1999b). Furthermore, Fe^{2+} induces both lipid mediated and lipid independent modifications of the ER membrane proteins as detected by the increase of fluorescence excitation (350–360 nm) and emission (440–450 nm) maximum and by the decrease of the intrinsic fluorescence of aromatic amino acid residue(s) (Kaplán et al. 2000).

The IP_3 receptor of Ca^{2+} channel, type I, is in rodent predominant and the best characterized neuronal form mainly enriched in cerebellum. Isoform II has only been detected in glia and type III is expressed mostly in basal ganglia and limbic system (Sharp et al. 1999). Calcineurin, a protein phosphatase, is also involved in the regulation of IP_3 receptor (Genazzani et al. 1999). Calreticulin, a Ca^{2+} binding protein of low affinity and high capacity, acts as a lumenal Ca^{2+} sensor for store depletion (Lehotský et al. 1993). Although calreticulin is widely distributed in the ER, it seems from the distribution of the IP_3- and ryanodine receptors that Ca^{2+} stores are not homogenous but rather molecularly heterogeneous. Rodent forebrain, as we detected by Western blot analysis, expresses proteins of the IP_3 receptor type I, SERCA isoform 2b and lumenal calreticulin. The isoforms of SERCA 3 and IP_3 receptor type II can be found only in cerebellum. The general response of neurons to ischemia seen on morphological level is associated with disturbed cellular integrity and ER aggregation, formation of cisternal stacks and appearance of cytoplasmic vacuoles (Nagata et al. 1999). In gerbil, transient ischemia/reperfusion did not affect the protein levels of SERCA 2b and calreticulin. However, cytoarchitecturally localized ischemia/reperfusion resulted after 7–10 days in a decreased amount of IP_3 receptor type I. Binding analysis and biochemical evaluation documented changes of both ryanodine- and IP_3 sensitive Ca^{2+} channels after ischemia- and seizure-induced cell death and other neuropathologies (Araki et al. 1998; Haughey et al. 1999; Pelletier et al. 1999). Furthermore, Nagata et al. (1999) have shown that basal Ca^{2+} uptake and caffeine induced Ca^{2+} release were normal in the ischemic region. However, in CA1 hippocampal sector the IP_3 induced Ca^{2+} release was inhibited and, as in our study, exhibited a loss of IP_3 receptor type I with the preservation of other IP_3 receptor isoforms. In addition, down-regulation of IP_3 receptor on both transcriptional and translational level has been documented in affected areas after IRI (Xia et al. 1998).

Using decapitation ischemia Parsons et al. (1999) have recently observed an inhibition of ER Ca^{2+} accumulation by uncoupling of Ca^{2+} uptake and ATP hydrolysis. In our experimental paradigm, both carotides occlusion caused depression of ER Ca^{2+} accumulation in pentobarbital anesthesia only with partial recovery after reperfusion. Surprisingly, under halothane anesthesia, significant changes were detected neither in the forebrain nor in the hippocampus. In addition, in line with Parson's results, decapitation ischemia led to significant changes of the Ca^{2+} uptake in both anesthetics used. These findings indicate that ischemic insult alters neuronal ER Ca^{2+} sequestration which is not due to inhibition of the Ca^{2+}-ATPase activity. However, the damaging effect depends both on the ischemic model and on the anesthetics.

In parallel, in the reperfusion period an ischemic insult induces a non specific alteration of physical properties of total membrane preparation such as modifications of proteins as well as content of lipoperoxidation products (Murín et al. 2001).

Conclusions

Reactive oxygen species are factors which can alter physical and functional properties of neuronal membranes *in vitro*. We suppose that disturbation of both plasma membrane and reticular ion transporters induced by ischemia can be explained at least partially by RO(N)S formation, and consequently it may participate in disturbation of neuronal ion homeostasis *per se* and may affect Ca^{2+} entry pathways. The alteration of number of Ca^{2+} transport proteins, such as PMCA, and/or IP_3 receptor during reperfusion period concomitantly with disturbed ion transport system can contribute to the changes which lead to derangement of Ca^{2+} homeostasis, and may participate and/or follow the delayed death of hippocampal neurons. Recent experiments suggest that calcium concentration is a discriminating factor for ischemic cell death execution or survival. State of relative calcium starvation can induce apoptosis, and *vice versa*, calcium overload leads to necrossis. New agents which can affect these signalling routes seem to be promising in preventing changes which culminate in selective neuronal loss after ischemia.

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