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MINIREVIEW

Molecular Pathways of Endoplasmic Reticulum Dysfunctions: Possible Cause of Cell Death in the Nervous System

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Summary

This review summarizes recent information on the role of calcium in the process of neuronal injury with special attention to the role of calcium stores in the endoplasmic reticulum (ER). Experimental results present evidence that ER is the site of complex processes such as calcium storage, synthesis and folding of proteins and cell response to stress. ER function is impaired in many acute and chronic diseases of the brain which in turn induce calcium store depletion and conserved stress responses. Understanding the mechanisms leading to ER dysfunction may lead to recognition of neuronal protection strategies.

Key words

Endoplasmic reticulum • Calcium store • Neuron • Damage

Introduction

One of the basic questions of neurosciences is to understand cellular and molecular processes which follow various injuries of the nervous system and lead to cell death. Neurons and glial cells with their unique biological properties together with vascular cells encounter progressive and/or regressive signals throughout the life of individual which may lead to maintained and/or improved cellular functions (plasticity) or trigger processes of injury and/or processes of acute/delayed cell death (Račay and Lehotský 1996).

Understanding the mechanism of injury and its temporal dependence within the cell can identify both the location and structures responsible for injury and also processes responsible for a tolerance/protective effect of various factors (Kristian and Siesjő 1998).

The role of calcium in neural cells

A physiological balance of ions is required for majority of cellular functions. On the other hand, factors which can damage neural cells may lead to ionic dysbalance and dysregulation of cytoplasmic and intra-organellar ionic homeostasis. Several biologically active substances including Ca^{2+} play a role of an important signal molecule in the cell (Berridge 1998). An increased Ca^{2+} concentration activates various Ca^{2+}-dependent physiological processes such as cell growth and development, synthesis and release of neurotransmitters, regulation of cellular excitability, activation of proteosynthesis and regulation of metabolism of saccharides and lipids (Kostyuk and Verkhratsky 1994). Due to an enormous difference of Ca^{2+} concentration between the extracellular (more than 1 mM) and the

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intracellular space (less than 1 μM) extracellular stimuli (hormones, neurotransmitters, growth factors, cytokines) can trigger fast increase of intracellular Ca^{2+} which can mediate cellular injury when Ca^{2+} intracellular overload occurs.

Thus, glutamate-induced Ca^{2+} entry is thought to be one of the major causes of neuronal death which follows ischemic/reperfusion injury of brain or cell damage in status epilepticus. It is also probably linked with neurodegenerative disease progression, such as Alzheimer´s disease or amyotrophic lateral sclerosis (Kristian and Siesjő 1998).

Resting low levels of Ca^{2+} and production of the Ca^{2+} signal within the cell is the result of interaction of two major transport routes:

a) Ca^{2+} entry from extracellular space and
b) interplay between the intracellular Ca^{2+} stores and Ca^{2+} binding mechanisms.

The balance between regulated Ca^{2+} entry and toxicity of the Ca^{2+} overload plays a major role in the evolution of cellular Ca^{2+}-dependent signal mechanisms activated by transduction of extracellular signals into the cell. The extracellular space of mammalian cells is practically an unlimited reservoir of Ca^{2+} which is regulated by interplay of various hormonal systems exchanging Ca^{2+} between the blood plasma, Ca^{2+} excretion and bones. Mechanisms of Ca^{2+} entry (Ca^{2+}-channels) have evolved for utilization of a stable Ca^{2+} ion gradient to generate a Ca^{2+} increase in the cell in the processes of signal transduction. As a rule, Ca^{2+} entry mechanism is operated by combination of several signals (membrane potential, hormones, neurotransmitters, growth factors). Low intracellular Ca^{2+} level (about 0.1 μM) is maintained by combination of its active extrusion and sequestration mechanisms, the protein of Ca^{2+} pump in the plasma membrane (PMCA) and the Na^{+}, Ca^{2+} exchanger. Sequestration is mediated by a protein of organelar Ca^{2+} pump (SERCA) into intracellular organels, mainly endoplasmic reticulum. Both pumps (PMCA and SERCA) utilize metabolic energy in the form of ATP (Lehotský *et al.* 2002, Garsia and Strehler 1999).

Role of endoplasmic calcium stores in neural cells

While the mechanism of Ca^{2+} entry by activation of ionotropic or metabotropic glutamate receptors and other Ca^{2+} channels has been genetically and pathophysiologicaly well described (Lehotský *et al.* 2001), the role of intracellular Ca^{2+} stores formed by endoplasmic reticulum (CER) and similar cellular components is still poorly understood (Szatkowski and Attwell 1994, Mody and MacDonald 1995). Why do cells that use stable and an almost unlimited extracellular Ca^{2+} reservoir require another signaling route of Ca^{2+} release from intracellular Ca^{2+} stores? The answer is based on following arguments (Berridge 1998). Ca^{2+} mobilization from internal stores is energetically cheapest way of increasing cytoplasmic Ca^{2+} and enables Ca^{2+} delivery effectively more deeply into the cellular interior. In comparison to Ca^{2+} entry, Ca^{2+} uptake into the ER provides faster recovery from increased Ca^{2+} levels to resting levels.

The majority of eukaryotic cells store an important part of intracellular Ca^{2+} in the ER. The volume of ER represents approximately 7.6 x 10^{-13} l/cell and its intramembranous Ca^{2+} concentration varies in the range between 2-5 mM (Corbett and Michalak 2000). The estimation of free Ca^{2+} concentration in the ER is proposed from the affinity of Ca^{2+} buffers, or from the experiments with recombinant Ca^{2+} binding proteins, and reaches the concentration of hundreds of μM to mM. Likewise, the gradient of Ca^{2+} concentration between the ER lumen is comparable to the gradient between the extracellular space and cytoplasm. Thermodynamically, the energy requirements for ion transport also depend on membrane potential. Thus, two biochemically similar Ca^{2+} pumps, the PMCA and the SERCA, transport different number of Ca^{2+} ions per 1 mol of ATP, the PMCA only 1 mol of Ca^{2+}/ATP, the SERCA up to 2 mol of Ca^{2+}/ATP. This means that cytoplasmic Ca^{2+} signal coming from ER costs only one half of energy compared to Ca^{2+}, which comes from extracellular space. In addition, the diffusion rate of Ca^{2+} in the cytoplasm is at least 30 times lower than in non-buffered medium due to the presence of high concentration and high capacity of cytoplasmic Ca^{2+}-binding proteins (buffers) (Berridge 1998). ER membrane contains two types of intracellular Ca^{2+} channels (receptors), i.e. one sensitive to plant alkaloid ryanodine and/or a second messenger, cyclic adenosine diphosphate ribose (cADPR), and another channel, which is stimulated by a lipidic messenger, inositol 1,4,5 trisphosphate (IP_3). Both channels share very high degree of structural similarity and both react to signals, which are produced in response to agonist (neurotransmiter, hormone), i.e. cADPR and IP_3. In addition, the diffusion rate of IP_3 and likely cADPR (more than 20 times higher than Ca^{2+} itself) enables very fast stimulation of Ca^{2+} release mechanisms by the

channel regulation from both luminal and cytoplasmic sides. This mutual receptor regulation represents a possibility for temporal and spatial organization of the cellular Ca^{2+} signal (Ghosh and Greenberg 1995). Evidence is accumulating that the total surface of ER in mammalian cells exceeds the surface of plasma membrane by more than 38 times. Thus, the ER is an ideal and a quite fast Ca^{2+} sink and store in the cell releasing Ca^{2+} into the cytoplasm (Račay and Lehotský 1996).

The amplitude and frequency modulation of the Ca^{2+} signal (wave) in the cell interior is regulated by binding of Ca^{2+} to Ca^{2+}-binding proteins and by its fast sequestration into the intracellular stores. Combination of these mechanisms with Ca^{2+} extrusion *via* PMCA or Na^{+}/Ca^{2+} exchanger decreases Ca^{2+} concentration to resting levels. In fact, Ca^{2+} buffering *via* Ca^{2+} binding proteins is not well designed to balance fast Ca^{2+} changes. However, several proteins of this group are indispensable for cellular life and are involved in the regulation of signal transduction (Corbett and Michalak 2000). Abnormally synthesized or metabolized Ca^{2+} binding proteins can be found in brain pathological states, such as Alzheimer´s disease, schizophrenia and epilepsy. In addition, the Ca^{2+} binding proteins can also be used as predictive marker in other pathologies e.g. melanoma (van Ginkel *et al.* 1998).

The role of endoplasmic reticulum in the pathological mechanisms of neural cell damage

Current progress in the elucidation of pathological mechanisms of neurological and psychiatric diseases is based on the application of novel technologies which identify molecular units responsible for the pathological processes. It has been shown in a number of studies that the cellular events mediating mainly necrotic death of neurons involve an excessive intracellular Ca^{2+} concentration, excitotoxic glutamate activity and production of reactive oxygen and nitrogen species (RO(N)S). (Kristian and Sjesjő 1998, Lipton 1999, Love *et al.* 2000). These factors are also believed to be the triggering pathogenic factors of several neuropathologies, such as the toxic effects of viruses, trauma, epileptic seizures and ischemia-reperfusion injury (Mattson *et al.* 2001, Sattler and Tymianski 2000). Mitochondrial electron transport is recognized a side-effect-producer of RO(N)S in the cell. Excessive formation of free radicals may attain an increased pathological significance when the mitochondrial membrane is damaged or permeability is altered (Simpson *et al.* 1998). Inducible isoforms of cyclooxygenase and NO synthase are other potential sources of RO(N)S. Likewise, produced RO(N)S can directly destroy biopolymers and can initiate the process of cellular death through aberrant activation of transcription factors (Lipton 1999). The experimental results also suggest that besides the acute RO(N)S effects, reactive species can trigger the process of delayed neuronal death even after apparent metabolic recovery (Dalton *et al.* 1999). Thus, it is not surprising that massive antioxidant treatment has a remarkable neuroprotective effect (Hicks *et al.* 1999, Baik *et al.* 1999).

In excessive concentrations, Ca^{2+} acts as a mediator of neuronal injury because it stimulates Ca^{2+}-dependent degradation processes, such as disaggregation of cytoskeleton, trigger of apoptosis, dysregulation of genetic information etc. (Lipton 1999, Toescu 1999). It has been shown in a number of studies that post-ischemic Ca^{2+} accumulation and/or molecular processes which can be triggered by this event are responsible for delayed neuronal death of selective neurons of hippocampus. However, it is not yet known which sources of Ca^{2+} and which signal routes are involved in this process (Szatkowski and Attwell 1994).

Despite the known unique role of ER in a) synthesis/folding/processing of newly formed membrane proteins, b) mechanisms of protein destined to secretion responses to the cellular stress, and c) the control of cellular Ca^{2+} homeostasis, its role in neuronal cell injury has been neglected until recently. This is probably the reason for disappointing trials to treat acute stroke by glutamate receptor inhibitors (NMDA receptor inhibitors) or calcium blockers in clinical settings (Love *et al.* 2000).

Evidence is accumulating on the mutual functional interplay between cellular organelles such as ER and mitochondria in response of neurons to various toxic agents, acute injuries and chronic pathological states of the brain, such as ischemia, epileptic seizures and Alzheimer's or Parkinson's disease. In order to carry out ER-based processes correctly the level of lumenal Ca^{2+} should be kept high enough. In addition, the proper lumenal oxidative environment is required for normal ER function. The fact that a disturbance of these processes is sufficient to cause cell damage indicates that they are crucial for normal cell functioning (Paschen and Frandsen 2001). In fact, it has been shown in neurons from worm *C. elegans* that high intracellular Ca^{2+} levels and release of ER-based Ca^{2+} stores are essential steps in the necrotic

death mechanism of neurons. Since ER-driven Ca^{2+} release has previously been implicated in mammalian necrosis, it has been suggested that necrotic death mechanisms may be evolutionary preconserved (Xu *et al.* 2001). The complex of strictly Ca^{2+}-dependent processes which take place in this subcellular compartment are, however, affected in different ways in various disorders. While the ER Ca^{2+} homeostasis is disturbed in ischemia, the ER-associated degradation of misfolded proteins is affected in Parkinson's disease and it is this unfolded protein response which is down-regulated in Alzheimer's disease (Mattson *et al.* 2001). It is now clear that low Ca^{2+} levels in the lumen of endoplasmic reticulum inhibit protein disulfide isomerases, causing malfolding of newly synthetized peptides in the ER lumen (Oliver *et al.* 1999).

Depletion of ER Ca^{2+} is induced in brain ischemia/reperfusion. Free arachidonic acid which is produced during ischemia induces inhibition of protein synthesis and mobilization (depletion) of Ca^{2+} from the ER (O´Neil *et al.* 1999). Kohno *et al.* (1997) have shown that after 5 min ischemia in the gerbil, content of ER calcium in vulnerable neurons is lost by 15 min reperfusion and is not recovered after 3 h of reperfusion. Furthermore, Ca^{2+} release from ER contributes to neuronal cell death because Ca^{2+} release blocker dantrolene can protect neurons against bioenergetic failure and cellular damage (Wei and Perry 1996). It has been shown in a series of studies from our laboratory that free radical generating systems depress Ca^{2+} uptake activity of isolated neuronal microsomes. The disruptive action of radicals involves membrane lipids concomitantly with membrane protein modifications (Račay *et al.* 1994, 1997, Lehotský *et al.* 1999, Kaplán *et al.* 2001). In rats, the occlusion of four blood vessels for 15 min induces oxidative damage of lipids and proteins as can be seen from the formation of lipid and protein oxidation products (Murín *et al.* 2001). Likewise, as proved by several laboratories including ours, the microsomes from postischemic brains display significant depression of the rate of Ca^{2+} accumulation (Parsons *et al.* 1999, Račay *et al.* 2000) that may be related to prolonged uncoupling of Ca^{2+} transport and ATPase activity and RO(N)S-mediated oxidative stress (Lehotský *et al.* 1999, Kumar *et al.* 2001).

As has been shown in cells from various tissues, the conditions associated with ER dysfunction induce a marked stress response, the unfolded protein response (UPR), the ER overload response and the ER associated protein degradation. Cellular response is also characterized by activation of the double stranded RNA activated protein kinase (PRK) and the PRK-like ER kinase (PERK). A central role is played by the chaperone GRP 78 by the activation of stress genes, as indicated by the observations that the overexpression of GRP 78 protects cells from death associated with ER stress. The unfolded protein response components activate either prosurvival mechanisms (e.g. by the activation of transcription of genes for GRP78, protein disulfide isomerase, and brain specific isoform of SERCA2b pump or proapoptotic mechanisms (e.g. by the activation of several kinases such as Jun N-terminal kinases, caspase-12 from the protease family and degradation of 28 S ribosomal RNA). In parallel, protein synthesis inhibition in neurons occurs during early reperfusion and involves alterations of initiation factors 2 (eIF2) and 4 (eIF4). Phosphorylation of the α subunit of eIF2 is catalyzed by transmembrane PER kinase (RERK) and inhibits the initiation of translation. PERK activation, along with depletion of endoplasmic reticulum Ca^{2+} store and inhibition of the ER Ca^{2+}-ATPase, SERCA2b, indicates that an ER unfolded protein response occurs as a consequence of brain ischemia and reperfusion. Brain ischemia and reperfusion also induce Ca^{2+}-dependent protease- μ-calpain-mediated or caspase-3-mediated proteolysis of eIF4 which can also promote apoptosis. Thus, alterations in eIF2 and eIF4 have major implications for protein synthesis in neurons during brain reperfusion and these pathways play a role in prosurvival and proapoptotic processes that may be differentially expressed in vulnerable and resistant regions of the reperfused brain (deGracia *et al.* 2002).

These facts suggest that any disturbance of intra-reticular (lumenal) processes is sufficient to trigger cellular damage. Thus, in contrast to the traditional calcium hypothesis which proposes that neuronal injury is related to the increased cytoplasmic Ca^{2+} activity (Kristian and Sjesjő 1998), recent studies suggest that neuronal cell injury might be related to both increase and decrease of cytoplasmic Ca^{2+} concentration (Sattler and Timianski 2000). A possible interpretation is that ER calcium store depletion *per se* rather than actual levels of cytoplasmic Ca^{2+} activity underlies the pathological process. ER calcium stores are evidently depleted when cytoplasmic calcium is low (Doutheil *et al.* 1999) and also under conditions when cell injury develops in the presence of high cytoplasmic Ca^{2+} activity (exposure to glutamate receptor agonists).

Various factors may induce ER dysfunction, e.g. a decrease of ER SERCA pump activity with age, metabolic and oxidative stress such as ischemia and

trauma, status epilepticus and also genetic defects – mutation of genes for proteins such as presenilin, or protein τ or amyloid protein in Alzheimer´s disease, protein of parkin in Parkinson´s disease leading to down-regulation or defects of the reticular protein folding and ER associated protein degradation (Paschen and Frandsen 2001).

Understanding of the exact mechanisms leading to ER dysfunction in different brain disorders may lead to recognition of selective therapeutical strategies. Novel pharmacological tools targeting signal routes which lead to selective loss or regeneration of neurons seem to be very prospective.

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