APOPTOSIS: AN IMPORTANT MECHANISM OF CELL DEATH IN CEREBRAL ISCHEMIA – THE ROLE OF GLUTAMATE

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Abstract:
One of the major leading causes of death and disability worldwide are the ischemic vascular insults to the brain (strokes). There are evidence which suggest that apoptosis plays an important role to cell death occurred in cerebral ischemia and reperfusion. Apoptosis is characterized by two different mechanisms, namely the extrinsic and the intrinsic pathways. Cerebral ischemia is characterized by an excessive release of glutamate at high concentrations within the core and penumbral region of the ischemic zone. Glutamate-induced neuronal cell death is associated with apoptosis through the increase of the Ca²⁺ concentration in cytosol and mitochondria.

Key words: intrinsic, extrinsic, NMDA, calcium, cytochrome c, mitochondria
In recent decades the incidence of cerebrovascular diseases has declined. However, vascular insults to the brain (strokes) remain the major leading cause of death and disability worldwide, in US being the third most common cause of death after heart disease and cancer. Cerebrovascular diseases can be divided into three categories: (a) parenchymal injuries associated with a generalized reduction in blood flow (including global hypoxic-ischemic encephalopathy), (b) infarcts caused by local vascular obstruction, and (c) hemorrhages within the brain parenchyma or the sub-arachnoid space. Usually, in practice stroke referred to a condition caused by the occlusion or hemorrhage of blood vessels supplying the brain, and is estimated to be responsible for approximately half of all hospitalizations for acute neurological disorders. Taking into account all the cerebrovascular diseases, the most common are infarcts accounting for approximately 80% of the total (1, 2). Strokes involve dysfunction or loss of brain cells and between global and focal cerebral ischemia there are significant differences in the mode of how cells are going to death. Basically, there are two fundamental types of cell death, apoptosis and necrosis and both of them are defined from the morphologically and biochemically point of view (3). Injuries associated with cerebral ischemia and reperfusion trigger multiple and overlapped cell signalling pathways which lead either to cell survival or cell damage. A lot of evidence suggest that apoptosis plays an important role beside necrosis to cell death occurred in cerebral ischemia and reperfusion (2). This article will present the morphological and biochemical aspects of apoptosis in cerebral ischemia, and also will analyse the place of glutamate and its links with the apoptotic processes.

APOPTOSIS
Apoptosis, term introduced in 1972 by Kerr et al., is often referred to as program cell death or cellular “suicide”, since death appears to result from induction of active processes within the cell itself, and represents a normal component of the development and health of multicellular organisms. It is an important mode of cell death through which the cell number during development is controlled and also the tissue homeostasis is maintained. Thus, apoptosis has a central role in several important physiologic processes, such as: (a) the programmed destruction of cells during embryogenesis, (b) the hormone-dependent physiologic involution, (c) the cell deletion in proliferating population, or (d) the deletion of autoreactive T cells in the thymus (1). The failure of cells to undergo physiologic apoptosis, in sense of its decrease or increase, can results in a variety of clinical disorders including cancer, autoimmunity, neurodegenerative diseases, hematopoietic disorders or infertility (3).

MORPHOLOGICAL FEATURES IN APOPTOSIS
In apoptosis the dying cells undergo rapid changes, which are reflected in both its structure and biochemistry, with distinctive morphology during the stages of the process. Usually, apoptotic process involves single cells but it is also possible to appear in clusters of cells. In hematoxylin and eosin stained sections, apoptotic cells appear as round or oval masses with intense eosinophilic cytoplasm. The nuclear chromatin shows to be condensed and aggregated in the periphery of the nucleus under the nuclear membrane, process which is termed as pyknosis. Condensed chromatin appears as well-delimitated masses of various shape and size. In the final stages of the process karyorrhexis occurs, most probably due to activation of endonucleases, being represented by a fragmentation of DNA into nucleosome-sized pieces. Cells rapidly shrink, form cytoplasmic buds, the plasma membrane bleb, and finally fragment into apoptotic bodies composed of membrane-bound vesicles of cytosol or organelles. These fragments are quickly extruded and phagocytosed or degraded, therefore even substantial apoptosis may be histologically unapparent (1, 4). Apoptotic cells often undergo plasma membrane changes such as loss of plasma membrane integrity and
translocation of phosphatidylserine located in the inner leaflet of the plasma membrane to the outer leaflet, as flow cytometry using annexin V has shown. This translocation facilitates the recognition of apoptotic cells by macrophages (3, 5, 6). Moreover, apoptosis does not elicit an inflammatory response, further hindering microscopic recognition (1).

**BIOCHEMICAL MECHANISMS OF APOPTOSIS**

Even though the exact mechanism by which apoptosis is initiated *in vivo* remains unclear, it is well known that the response is trigger-dependent and specific for the cell type studied (3). Nowadays, numerous apoptosis-related genes and their gene products have been identified. The two modern laboratory methods, transcriptomics (the measure of the quantity of messenger RNA present in a cell at a particular time) and proteomics (the characterization of proteins including posttranslational modification and subcellular distribution) have been used to evaluate the genes involved in apoptosis, both its initiation and cell survival. Research proves that there dose exist six major protein domains (repeating structural elements) associated with mammalian apoptosis and over 100 genes in the human genome have been reported within these six groups: caspase (cysteine dependent protease which cleaves at aspartate residues) catalytic domains, caspase-associated recruitment domains (CARDs), death domains (DDs), death effector domains (DEDs), BIR domain (IAPs) and Bcl-2 homology (BH) domains (3, 5).

Trigger signal transduction in apoptosis may occur by two distinct pathways: extrinsic or receptor-linked apoptosis respectively intrinsic or mitochondria-mediated apoptosis. Even though these two pathways are viewed as separate mechanisms, capable for independent functioning, cross-talk can occur between them at different levels based of the type of apoptosis-modulating proteins expressed (5).

**EXTRINSIC APOPTOTIC PATHWAY – THE DEATH RECEPTOR PATHWAY**

The extrinsic apoptotic pathway is a receptor-linked mechanism which requires the binding of a ligand to a death receptor located on the cell surface. Death receptors implicated in the extrinsic pathway belong to the tumor necrosis factor receptor (TNFR) super family, including TNFR1 (p55, CD120a), Fas receptor (CD95, Apo1), DR3 (Apo 2, Weasle), DR4 (TrailR1), DR5 (TrailR2) and DR6. All of these receptors contain the death domain (DD) in their structure, more precisely in their intracellular region, which is implicated in the recruitment of downstream cellular apoptotic proteins (2, 7). The Fas (Apo-1/CD95) receptor is a prototype death receptor which has become paradigm for the study of death receptor-mediated apoptosis (2).

Soluble molecules belonging to the Tumor Necrosis Factor (TNF) family, such as tumor necrosis factor-α, Fas ligand (Fas-L), TNF-related apoptosis inducing ligand (TRAIL), which are physiologically secreted as homotrimers, bind to plasma membrane receptors of the TNF-Receptor (TNFR) family causing their trimerization and the subsequent activation (8). Upon the binding to the trimeric ligands, death receptors form micro aggregates at the cell surface. This receptor aggregation allows to a multiple step mechanism for recruitment of the downstream DD-containing adaptor molecules, Fas-associated death domain (FADD) and eventually the TNFR type 1-Associated Death Domain protein (TRADD), to the cytosolic tail of the receptor. The interaction between receptor and the FADD molecule is based on the homotypic contact, both of them containing death domains (DDs). FADD contains also a death effector domain (DED) in its N-terminal region which allows the interaction with the N-terminal tandem DEDs of procaspase-8 and -10 and c-FLIP_L/S/R, interaction based also on the homotypic contact. The multiprotein complex formed by oligomerized (most probably trimerized) receptors, the DD-containing adaptor molecule FADD (Fas-associated death domain), 2 isoforms of procaspase-8 [procaspase-8/a (FLICE, MACHα1, Mch5) and...
procaspase-8/b (Macha2), procaspase-10, and the cellular FLICE-inhibitory proteins (c-FLIPL/S/R) is referred as Death-Inducing Signaling Complex (DISC). The DISC formation results in assembly of procaspase-8 and -10 molecules in close proximity to each other, allowing activation of them to initiator caspases-8 and -10 (2, 8, 10, 11, 12) (Figure 1). Subsequent activation of initiator caspases-8 and -10 leads to the cleavage process of the effector caspases -3, -6, and -7, which are responsible by the further processes and enzymatic cascades that will conduct to the apoptotic cell death (8).

The extrinsic apoptotic pathway may utilize cytochrome c release as well. In some situations, caspase-8 could cleave proapoptotic Bid protein, a member of the BH3-only domain subgroup of the Bcl-2 family, into a truncated form tBid, which translocates to mitochondria where, through the binding with Bak, determines the release of cytochrome c from the mitochondrial intermembrane space (2, 3).

There are data which suggest that the receptor-mediated activation of caspases may participate in ischemic brain damage. The activation of caspase-8 has been proved in studies on experimental brain ischemia. The release of TNF-α by neurons and glia, in association with the upregulation of Fas mRNA and protein levels, have been also observed in the vulnerable areas after hypoxic ischemic injury. Moreover, a marked increase in the Fas-L expression is found in the penumbral region during early reperfusion after middle cerebral artery occlusion (MCAO). These strongly data suggest an upregulation of death receptors which lead to assembly of DISC and the activation of procaspase-8 after focal cerebral ischemia (2).

**Figure 1.** Scheme of procaspase-8 processing at the CD95 DISC. CD95 DISC formation is triggered by extracellular cross-linking with CD95L, which is followed by oligomerization of the receptor. FADD is recruited to the DISC by DD interactions; procaspase-8 and -10 as well as c-FLIP proteins are recruited to the DISC by homophilic DED interactions. Upon recruitment to the DISC, procaspase-8 undergoes processing by forming dimers. (A) The first step of procaspase-8 cleavage occurs between 2 protease subunits. As a result of the first cleavage step the p10 subunit is formed, which is not released into the cytosol but remains bound to the DISC as it is involved in the interactions with the large protease subunits. (B) The second cleavage step takes place between the prodomain and the large protease subunit at Asp216. As a result of this cleavage step the active caspase-8 heterotetramer is formed, which is then released into the cytosol. (C) Prodomain p26/p24 remains bound to the DISC (11).

**INTRINSIC APOPTOTIC PATHWAY – THE MITOCHONDRIAL PATHWAY**

Mitochondria which are involved in many physiological processes essential for cell survival, such as energy production, redox control, calcium homeostasis, and also metabolic and
biosynthetic pathways, play an essential role in physiological cell death mechanisms. These genetically controlled mitochondrial pathways are often subject to dysfunction, and therefore mitochondria can be central players in some pathological conditions such as cancer, diabetes, obesity, ischemia/reperfusion injury, and neurodegenerative disorders like Parkinson and Alzheimer diseases (13).

Mitochondrial outer membrane permeabilization (MOMP) is considered to be the “point of no return” in the mitochondrial apoptotic pathway. Regarding the mechanisms of MOMP, there are two principal hypotheses (Figure 2). The first one considers that MOMP is a process that is essentially intrinsic to the outer membrane, regulated by the Bcl-2 family of proteins which can promote or prevent the formation of pores. The Bcl-2 family of proteins includes both proapoptotic and antiapoptotic members, each containing one or more Bcl-2 homology domains (BH1, BH2, BH3, and BH4). Antiapoptotic members such as Bcl-2, Bel-xL, and Mcl-1 contain all four BH domains. The proapoptotic members are divided into two subgroups. The first subgroup comprises the Bax, Bak, and Bok proteins, which possess BH1, BH2, and BH3 domains. The second one consists of the most numerous BH3-only proteins, including Bid, Bad, Bim, Puma, and several others. Even though several proteins of Bcl-2 family possess ion channel activity in lipid bilayers, only the multidomain proapoptotic proteins Bax and Bak can render membranes permeable to cytochrome c or for larger macromolecules. In nonapoptotic cells Bax and Bak both seem to be in an inactive state, Bax being mostly free in the cytosol while Bak is constitutively localized in membranes of the mitochondria and endoplasmic reticulum. Throughout a not completely understood activation process, Bax oligomerize and insert stably into the mitochondrial outer membrane (MOM), forming pores which result in either a decrease in the inner mitochondrial transmembrane potential ΔΨm or in the opening process of the voltage-dependent anion channel (VDAC) with a consequently release of cytochrome c from the space between the inner and outer mitochondrial membranes (IMS). The BH3-only proteins Bim or Bid are the “direct activator” proteins which trigger the MOMP mediated by Bax and Bak. However, BH3-only proteins fall into two functional classes: the direct activators Bim and Bid mentioned above, and the others, which act as “derepressors” by binding competitively to the antiapoptotic family members and thus freeing up the direct activator proteins to induce Bax/Bak-mediated MOMP. Other proteins of the MOM could modulate or potentiate the function of Bax and Bak. Thus, a number of proteins unrelated to the Bcl-2 family can interact with Bax, either inhibiting or enhancing its activation. Similarly, Bak has been reported to associate with VDAC2 at the mitochondrial membrane, and this association inhibits the oligomerization and proapoptotic activity of Bak. The nuclear transcription factor p53, which is activated through the phosphorylation of serine 46 by homeodomain-interacting protein kinase-2, induces gene transcription when it is upregulated, including Bax. However, the p53 protein can also directly induce Bax-mediated membrane permeabilization in cells, although it lacks a clearly identifiable BH3 domain (3, 13).

The second hypothesis for MOMP is based on a phenomenon known as the mitochondrial permeability transition (PT) which involves the rapid permeabilization of the inner mitochondrial membrane (IM) to solutes smaller than 1.5 kDa through formation of the permeability transition pore (PTP) (3, 13). In healthy cells, the inner mitochondrial membrane (IM), which separates the intermembrane/intercristae space from the matrix, is almost impermeable to all ions, here including also protons. Therefore, complexes I–IV of the respiratory chain, based on this property of the IM, build up the proton gradient that is required for oxidative phosphorylation. The complex V of the respiratory chain uses this proton gradient to drive ATP synthesis. Therefore, the maintenance of the proton gradient becomes of vital importance for cellular bioenergetics. A charge imbalance results from the generation of this electrochemical gradient across the IM, and that forms the basis of the
inner mitochondrial transmembrane potential $\Delta \Psi m$. Even though a transient loss of the $\Delta \Psi m$, through the “flickering” of one or several IM pores, may occur in physiological circumstances, permanent or long-lasting $\Delta \Psi m$ dissipation is often associated with cell death, and for that reason all constituents of the mitochondrial matrix and all metabolites, through highly selective channels and transport proteins, cross the IM in a very controlled mode (14). The PTP complex is a “megapore” which is thought to span the contact sites between the inner and outer mitochondrial membranes. It is considered to be composed of at least three proteins, namely the voltage-dependent anion channel (VDAC) located in the outer membrane, the soluble matrix protein cyclophilin D (Cyp D), and the adenine nucleotide translocase (ANT) which is located in the inner mitochondrial membrane. Regarding the ANT protein, its presence in the structure of PTP is controversial. Depending on the level of Ca$^{2+}$ PTP can show two different levels of conductance. Under normal calcium homeostasis, the PTP exists in a state of low conductance but when excess Ca$^{2+}$ is released from the endoplasmic reticulum and overloads the mitochondria, the pore commutes to a high-conductance state. This irreversible passage from low to high conductance strictly depends on the saturation of the calcium-binding sites of the PTP. The high conductance conformation allows free diffusion of water and ions between the cytosol and the matrix, causing collapse of the inner membrane gradient $\Delta \Psi m$, uncoupling of oxidative phosphorylation, and swelling of the mitochondrial matrix. This leads to rupture of the MOM and consequent release of proteins from intermembrane space (IMS). A few proapoptotic stimuli, such as calcium overload, oxidative stress, or ischemia/reperfusion, seem to mediate cytochrome $c$ release directly through the mitochondrial permeability transition (PT). This release of mitochondrial cytochrome $c$ often occurs after a decrease in the inner mitochondrial transmembrane potential $\Delta \Psi m$ but mitochondrial depolarization does not always precede the release of cytochrome $c$ and is often blocked by caspase inhibition. In this second case the loss of inner mitochondrial membrane gradient $\Delta \Psi m$ does not result from mitochondrial permeability transition (PT), but rather from the caspase dependent cleavage of at least one substrate in the respiratory chain, the p75 subunit of complex I. Cleavage of p75 disrupts consequently oxygen consumption, increases ROS production, and is one of several events that may preclude recovery for the cell. Moreover, due to the fact that the mitochondrial matrix swelling is not always observed in apoptotic cells the generality of PT as a primary mechanism for MOMP and apoptosis is controversial (3, 13).

Mitochondrial outer membrane permeabilization (MOMP) conducts to the release of multiple proteins from the mitochondrial intermembrane space (IMS) into the cytosol. This leads to caspase activation in the cytosol, loss of mitochondrial transmembrane potential $\Delta \Psi m$, cellular ATP depletion, and free radical production. The main proteins released from the mitochondrial intermembrane space are cytochrome $c$, Smac/Diablo protein, endonuclease G (Endo G), apoptosis-inducing factor (AIF), HtrA2/Omi, Nipsnap 3 and 4, glutamate dehydrogenase (Gdh), CLPX (a regulatory component of mitochondrial Clp protease – CLPP), LRPPR (leucine-rich pentatricopeptide repeat motif-containing protein), and 3-hydroxyisobutirate dehydrogenase. Cytochrome $c$, one of the released IMS proteins, is an important component of the mitochondrial respiratory chain. The intrinsic apoptotic pathway is mediated mainly by the mitochondrial release of cytochrome $c$ and it is often activated by damaged DNA when that is not sensed and repaired by checkpoint genes. When translocated into the cytoplasm, cytochrome $c$ stimulates the assembly of a multiprotein complex known as the Apaf-1 apoptosome, composed of apoptotic protease activating factor-1 (Apaf-1), procaspase 9 and either ATP or dATP. The last step of the apoptotic program is DNA degradation which is carried out by various nucleases. The caspases are an apoptosis-related family of proteases that, upon activation, cleave specific substrates, leading to the demise of the cell. Thus, caspase-9 is recruited to the apoptosome and activated, initiating a cascade of
effector caspase activation. However, activated caspase 9 is not necessary for the apoptosome to recruit procaspase 3 and activate it by cleavage to caspase 3. Active caspase 3, finally, mediates the DNA fragmentation into 180–200 base-pair units and multiples of these units. This DNA fragmentation is initiated by caspase 3 activation of inactive caspase-activated deoxyribonuclease by removing its inhibitor. Caspases are controlled by specific cellular inhibitors called Inhibitor of Apoptotic Proteases (IAP), which can bind to them, thus blocking their function. To counteract Inhibitor of Apoptotic Proteases (IAP) function, Smac (second mitochondrial activator of caspases)/Diablo (direct IAP binding protein with low pI) and HtrA2/Omi are released from mitochondria in order to bind to or, respectively, to cleave IAPs. However, the intrinsic pathway may also operate via caspase-independent mechanisms. These mechanisms involve the release from mitochondria and translocation into the nucleus of at least two proteins, namely apoptosis inducing factor (AIF) and endonuclease G (Endo G). Endonuclease G is released from mitochondria to the nucleus, where it cleaves DNA. The nuclear effects of apoptosis-inducing factor (AIF), which is also translocated from mitochondria to the nucleus, include chromatin condensation and formation of high-molecular-weight DNA fragments (3, 4, 13, 14, 15, 16, 17). The activation of caspase 3 is followed by the translocation of phosphatidylserine located in the inner leaflet of the plasma membrane to the outer leaflet, thus facilitating the recognition of apoptotic cells by macrophages (3, 13).

Figure 2. Molecular mechanisms of mitochondrial outer membrane permeabilization (MOMP). There are two proposed models of MOMP, both leading to cytochrome c release: (i) The formation of Bax pore: Bax or Bak forms a pore in the MOM after activation by a BH3-only protein such as Bid. (ii) The opening of the permeability transition pore (PTP): the opening of the PT pore allows an influx of water and ions into the matrix, causing matrix swelling; this leads to rupture of the MOM, releasing IMS proteins such as cytochrome c. MIM, mitochondrial inner membrane; PBR, peripheral benzodiazepine receptor (13).

GLUTAMATE EXCITOTOXICITY AND APOPTOSIS IN CEREBRAL ISCHEMIA
Glutamate is the major excitatory neurotransmitter in the mammalian brain and also a key mediator of intracellular communication, plasticity, growth and cell differentiation. The extracellular concentration of glutamate under normal physiological conditions is maintained in the micromolar range. Glutamate is responsible for initiation of postsynaptic signalling through distinct ionotropic and metabotropic glutamate receptors. There are three types of ionotropic glutamate receptors, namely, N-methyl-D-aspartate receptors (NMDARs), 2-
amino-3-(3-hydroxy-5-methylisoxazol-4-yl) propionate receptors (AMPARs), and kainate receptors. Each of these ionotropic receptors has several subtypes. Activation of ionotropic receptors leads to an enhanced permeability to sodium (Na⁺), potassium (K⁺) and/or calcium (Ca²⁺) ions in the associated ion channels. NMDARs are made from three major subfamilies of subunits, which are the ubiquitously expressed NR1 subunit, four distinct NR2 subunits (A–D), and two NR3 members (A and B). Highly active, functional NMDARs contain both NR1 and NR2 subunits and in some cases NR3 subunits. NMDA receptors, which are highly permeable to Ca²⁺ and Na⁺, seem to be slow gating channels. Ion permeability contributes to membrane depolarization, and the influx of exogenous Ca²⁺ generates the intracellular calcium transients that are responsible for the physiological effects of NMDAR signalling. The C-terminal domains of NMDAR subunits mediate NMDAR interactions with multiple intracellular synaptic and cytoskeletal proteins, thus forming large receptor-linked multiprotein complexes in the postsynaptic density (PSD), in which NR2B subunit of NMDAR binds to PSD-95/SAP90, chapsyn-110/PSD93, and other members of the membrane-associated guanylate kinase (MAGUK) family. Such protein–protein interactions link NMDA receptors to distinct downstream signalling molecules, including distinct downstream signalling pathways involved with neurotoxicity. AMPA receptors (AMPARs) mediate the fast excitatory component of glutamate neurotransmission and are tetrameric NMDAR subunits assembled from combinations of GluR1–GluR4 (GluR-A–GluR-D) subunits. AMPARs are ionotropic receptors permeable to K⁺ and Na⁺ but are normally impermeable to Ca²⁺. Some AMPA channels show to be calcium ions permeable and emerging evidence supports the idea that these unusual channels, which are preferentially expressed on discrete populations of neurons, might be crucial contributors to injury in ischemia. Ca²⁺-permeable AMPARs also exhibit larger Na⁺ currents which correlate with their capacity to elicit neurotoxicity. The number of Ca²⁺ permeable AMPA channels is regulated both in responses to physiological synaptic activity and in certain pathological states. For example, whereas relatively few Ca²⁺ permeable AMPA channels are normally present on hippocampal pyramidal neurons (HPNs), the number of these channels can increase sharply after ischemia. Finally, kainate receptors are comprised of subunits that are homologous in transmembrane topology and stoichiometry to AMPA and NMDA receptor subunits. Kainate receptor subunits can be further divided into two groups based on their high affinity (KA1 and KA2) or lower affinity (GluR5-7) binding to kainic acid. KA2 was shown to bind PSD95/SAP90 and SAP102, postsynaptic density (PSD) proteins that may participate in the regulation and localization of kainate receptors, but which may also have a role in excitotoxic cell death. Glutamate metabotropic receptors mediate their actions through GTP-binding protein-dependent mechanisms and determine mobilization of Ca²⁺ from internal stores (18, 19).

Cerebral ischemia (stroke) triggers a complex series of biochemical and molecular mechanisms that impairs the neurologic functions through breakdown of cellular integrity mediated by excitotoxic glutamatergic signalling, ionic imbalance, free-radical reactions, nitric oxide and other factors. Nowadays it is known that following cerebral ischemia there is an excessive release of glutamate at high concentrations within the core and penumbral region of the ischemic zone. The breakdown of astrocytic functions results into impaired glutamate uptake by astrocytes. It has been reported recently that glutamate-induced neuronal cell death is associated with apoptosis, based on the characteristic fragmentation of DNA, morphological changes of the cell, activation of calpain and caspase-3, as well as the upregulation and/or translocation of apoptosis inducing factor (AIF) from mitochondria into cytosol and nuclei. These data suggest that glutamate, at higher concentrations, may induce apoptosis through caspase-dependent and caspase-independent mechanisms. Moreover, inflammatory reactions initiated at the neurovascular interface combined with the alterations
in the dynamic communication between the endothelial cells, astrocytes and neurons are thought to substantially contribute to the pathogenesis of the disease (2, 19).

Despite the ubiquitous presence of Ca\textsuperscript{2+} ions in cells, different physiological Ca\textsuperscript{2+}-dependent processes, including synaptic plasticity and gene expression, are separately regulated through distinct signalling pathways and are linked to specific routes of Ca\textsuperscript{2+} influx. Several routes of calcium ion entry into the cells exist and many of them are dysregulated after brain ischemia, anoxia or excitotoxicity. Calcium ions can gain entry into the neurons through several mechanisms which include the already mentioned activation of glutamate receptors (NMDA, AMPA or KA) or through a range of channels and transporters (TRPM2, TRPM7, NCX, ASICs, CaV1.2, and hemichannels). Nevertheless, calcium can also be released from the internal stores. The release of calcium from internal stores is mediated through the glutamate metabotropic receptors. The subsequent binding of glutamate to ionotropic N-methyl-D-aspartate (NMDA) receptors, 2-amino-3-(3-hydroxy-5-methylisoxazol-4-yl) propionate receptors (AMPA) and kainate receptors (KA) promotes Ca\textsuperscript{2+} influx, which in pathological conditions becomes excessive triggering the intrinsic way of apoptosis by enhancing the permeability of PTP. Therefore, a range of downstream proteases and phospholipases that degrade membranes and proteins essential for cellular integrity, causing in this way a subsequent cell death, are activated. In pathological conditions calcium can be released from the internal stores either through physical damage of mitochondria and the endoplasmic reticulum, or a malfunction of receptors and channels which are present in their membranes (2, 18).

The ischemic/reperfusion injury leads to depletion of energy stores which determines altered cell function by interrupting adenosine tri-phosphate (ATP)-dependent process, predominantly, the sodium/potassium ATPase (Na\textsuperscript{+}/K\textsuperscript{-}-ATPase). Thus, the disruption of ionic gradients across the membranes is a consecutive event to the dysregulation of Na\textsuperscript{+}/K\textsuperscript{-}-ATPase. The extracellular K\textsuperscript{+} increases in association with an influx of Na\textsuperscript{+}, chloride (Cl\textsuperscript{-}), and Ca\textsuperscript{2+} into the cells, processes which determine the membrane depolarization and reversal of the amino acid transporters. Under these conditions, both voltage-operated and receptor-operated Ca\textsuperscript{2+} channels are activated, leading to an elevation of free cytosolic Ca\textsuperscript{2+}. Such high increase of cytoplasmic Ca\textsuperscript{2+} concentration can triggers a range of downstream neurotoxic cascades, including mitochondrial Ca\textsuperscript{2+} overload with the inhibition of ATP production, breakdown of phospholipids, proteins, and nucleic acids by activation of Ca\textsuperscript{2+}-dependent enzymes such as calpains and other proteases, phospholipases, protein kinases, nitric oxide synthase (NOS), calcineurin and endonucleases. As a consequence of both reversal of glutamate transporters and Ca\textsuperscript{2+}-dependent exocytosis a massive release of excitatory amino acids, particularly glutamate, may appear. The augmented intracellular Ca\textsuperscript{2+} further promotes increase in extracellular glutamate, thus, propagating the excitotoxicity. Studies have demonstrated that activated caspase-3 cleave and inactivate the plasma membrane Ca\textsuperscript{2+} pump (PMCA) in neurons and non neuronal cells undergoing apoptosis, which results in further intracellular Ca\textsuperscript{2+} overload. Moreover, during brain ischemia, in neurons undergoing excitotoxicity calpains cleave NCX which is the major plasma membrane Ca\textsuperscript{2+}-extruding system. Experimental data show that the overexpression of calpastatin, an endogenous calpain inhibitor, or the expression of NCX2 isoform, which is not cleaved by calpains, prevents Ca\textsuperscript{2+} overload and protects neurons from excitotoxic cell death. Conversely, suppression of NCX3 by RNA interference sensitizes neurons to Ca\textsuperscript{2+} overload and excitotoxicity. Moreover, lethal Ca\textsuperscript{2+} signalling by NMDARs is determined by the molecules with which they interact. The suppression of PSD95 using antisense oligonucleotides leads to uncouple NMDAR-mediated signalling from nitric oxide neurotoxicity. Through the suppression of PSD95 by antisense oligonucleotides or the disruption of the interaction between NMDARs and PSD95 using specific peptides, the Ca\textsuperscript{2+}-activated nitric oxide
production by NMDARs is selectively blocked, without affecting nitric oxide synthase expression or function. Through these methods the excitotoxic cell death is blocked without blocking NMDAR currents, and thus the excitotoxicity is prevented without the negative consequences associated with NMDAR inhibition. Even though it is widely accepted that intracellular Ca\(^{2+}\) overload after ischemia is mainly mediated through glutamate-gated channels, the failure of glutamate antagonists in clinical trials regarding neuroprotection proved the existence of glutamate-independent mechanisms of Ca\(^{2+}\) entry. Acidosis is a common feature of brain ischemia and the low pH which occurred in the ischemic tissues is assumed to play an important role in the pathological processes. The mechanisms related to how acidosis leads to ischemic brain injury remains unclear. It has been demonstrated that activation of Ca\(^{2+}\)-permeable acid-sensing ion channels (ASICs), in particular the ASIC1a subunit, is mainly responsible for acidosis-mediated, glutamate receptor-independent, ischemic brain injury. Acid-sensing ion channels (ASICs) composed of ASIC1a subunit exhibit a high Ca\(^{2+}\) permeability and play important roles both in the physiologically synaptic plasticity and pathologically acid induced cell death. Ischemia and low pH enhances ASIC currents through the phosphorylation at Ser478 and Ser479 of ASIC1a, leading to an exacerbated ischemic cell death. The phosphorylation is catalyzed by Ca\(^{2+}\)/calmodulin-dependent protein kinase II (CaMKII), as a result of activation of NR2B containing N-methyl-D-aspartate subtype of glutamate receptors (NMDARs) during ischemia. In CNS neurons, NR2B-NMDARs are localized predominantly at extrasynaptic sites and are capable of detecting extrasynaptic spillover of glutamate. Because these extrasynaptic NMDARs are known to trigger cell death pathways, the specific coupling of NR2B-ASIC1a suggests that an excessive glutamate release caused by ischemia may activate extrasynaptic NR2B-NMDARs, leading to exacerbated ASIC1a-mediated acidotoxicity. The specific coupling between NR2B subunit of NMDA receptor and ASIC1a through Ca\(^{2+}\)/calmodulin-dependent protein kinase II has suggested a new direction for therapeutic strategies against excitotoxic and acidosis mediated ischemic damage. Specific blockade of NMDAR/CaMKII-ASIC coupling may reduce neuronal death after ischemia and also other pathological conditions involving excessive glutamate release and acidosis. Therefore, NR2B-specific antagonist, CaMKII inhibitor, or overexpression of mutated form of ASIC1a with Ser478 or Ser479 replaced by alanine (ASIC1a-S478A, ASIC1a-S479A) in cultured hippocampal neurons prevented ischemia-induced enhancement of ASIC currents, cytoplasmic Ca\(^{2+}\) elevation, as well as neuronal death. These data indicate that combined inhibition of both glutamate-mediated excitotoxicity and ASIC1a-mediated acidosis may prove to be a novel neuroprotective strategy for stroke patients. Besides the activation of signalling mechanism involving calcium/calmodulin-dependent kinases (CaMKs), during ischemia the mitogen-activated protein kinases (MAPKs) such as extracellular signal-regulated kinase (ERK), p38, and c-Jun N-terminal kinase (JNK) are also stimulated. These transducers are distributed in such fashion in order to bring them in contact with appropriate molecular targets conducting to altered gene expression, e.g. ERK and JNK mediated early gene induction, responsible for activation of cell survival/damaging mechanisms (2, 18, 19, 20).

In addition to the increased Ca\(^{2+}\) influx and cellular concentration, ionotropic glutamate receptors promote also an excessive influx of Na\(^{+}\) with concomitant cell swelling and oedema. Imbalances in other ions are also important during ischemia, such as zinc (Zn\(^{2+}\)). Large amounts of zinc (Zn\(^{2+}\)) are stored in vesicles of excitatory neurons and released simultaneously upon depolarization (2).

In conclusion, there is an indispensable need to develop cellular and molecular research in this vital area of cerebral ischemia in order to generate important information regarding the underlying mechanisms of cellular survival/damage in order to develop suitable and effective neuroprotective strategies.
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