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Molecular mechanisms involved in cerebral ischemic-induced neuronal death

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Abstract

The understanding of the cellular, molecular and biochemical processes that take place during the production of brain lesions has increased recently due to numerous studies of microscopy, cellular and molecular biology, performed on both human material and experimentally on a laboratory animal. Although new therapeutic methods have emerged to treat strokes and prevent them, due to the complexity of morpho- and pathophysiological processes, the beneficial effects of treatment are still far from satisfactory.

Keywords: ischemic stroke, neuronal death, excitotoxicity, glutamate, free radicals

INTRODUCTION

In recent years, many research teams have addressed strokes both clinically and paraclinically, as strokes are currently a major public health problem worldwide.

This explains why considerable efforts are being made worldwide for a more in-depth knowledge of the etiopathogenic and morphopathological phenomena that contribute to the occurrence of strokes, as well as ways to prevent and treat these afflictions [1-7].

Ischemic stroke occurs due to the reduction or suppression of the blood supply to an area of the brain. This phenomenon initiates a cascade of events called an "ischemic cascade" in which the brain tissue in that area reduces its activity or ceases to function [8]. The lack of oxygen for about 60 to 90 seconds can cause reversible neuronal damage, but if it lasts longer, irreversible damage can occur, leading to brain tissue death, a condition characterized histopathologically as a cerebral infarction [9].

The appearance of hypoxia or anoxia triggers a series of interdependent events that lead to the appearance of cellular lesions (neuronal and glial) and even to their death.

Molecular changes are interconnected, complex, making it difficult to predict their relative pathogenic importance in different models of ischemia. In particular, the molecular changes induced by transient focal ischemia are not equivalent to the changes that occur in the core or in the penumbra area in permanent ischemia. Therefore, the relative contribution of the different lesion mechanisms in different types of ischemia is extremely difficult to assess.

For example, the direction of the evolution of a neuron located in the ischemic penumbra area, towards survival or apoptosis, is influenced by the existing ROS/RNS concentration through functional proteins [10,11].

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The paper is a review using four international databases (PubMed/MedLine, Web of Science, Scopus-Elsevier, and ScienceDirect) for ischemic neuronal death. A documentation of the published scientific articles using the following key words: "ischemic stroke", "neuronal death", "oxidative stress", "reactive oxygen species (ROS)", "glutamate", and "calcium ions (Ca^{2+})" was carried out.

THE POSSIBLE MOLECULAR PATHWAYS INVOLVED IN ISCHEMIC NEURONAL DEATH

Relatively recent data identified several mechanisms by which changes in the cerebral parenchyma occur. These appear to be: acidotoxicity, excitotoxicity, calcium toxicity, free radical toxicity, nitric oxide toxicity, inhibition of protein synthesis, mitochondrial disorders, triggering of local inflammatory process, formation and expansion of cerebral edema etc.

Acidotoxicity is manifested during the onset of ischemia by drastically reducing oxygen and activating anaerobic glycolysis, when an accumulation of lactic acid occurs, which, depending on the severity of the ischemia, blood glucose levels and the degree of ATP hydrolysis, causes a decline in the intracellular pH at levels between 6.5 and 6.0 or even lower. Because the severity of ischemic lesions correlates with the degree of acidosis, the idea that acidosis is neurotoxic has been postulated [12,13].

Excitotoxicity occurs shortly after the onset of ischemia, when a number of excitatory and inhibitory neurotransmitters are released, resulting in the activation of their specific receptors. Among these neurotransmitters, special attention has been given to glutamate, which at high concentrations is known to produce excitotoxicity [14,15].

The pathophysiological role of glutamate

A major cause of neuronal damage is the release of the excitatory neurotransmitter, glutamate. The concentration of glutamate outside the nervous system cells is kept low by the so-called absorption transporters which are fed by ion concentration gradients (mainly Na^+) in the cell membrane. Blocking the glutamate transport systems, which carry the molecule from the outside of the cells to the inside, leads to the appearance of a large amount of glutamate in the extracellular space. Glutamate acts on the receptors in nerve cells (special NMDA receptors), which, in turn, produce an influx of calcium that activates enzymes which digest cellular proteins, lipids and other nuclear materials.

Glutamate is one of the major excitatory neurotransmitters in the central nervous system. It controls various cellular and synaptic functions, cell death, survival, motor functions, learning and memory [16]. Its level in the mammalian brain is 1,000 times higher than other important neurotransmitters, such as dopamine or serotonin. Although glutamate has important physiological functions in the central nervous system, it has been suggested that it takes part in the pathophysiological processes of many diseases, such as epilepsy, neurodegenerative diseases and stroke [17].

Glutamate is not synthesized in neurons, neither is it taken from the circulation [18], but it is synthesized in astrocytes, through an intermediate metabolite, glutamine [19,20].

Neurons are normally exposed to small, transient, glutamate-induced impulses due to efficient absorption and destruction mechanisms of the glutamate in the synaptic space, but neurons are subject to excessive glutamatergic stimulation during ischemia due to high levels of extracellular glutamate, with hyperstimulation of its receptors [21,22].

The concept that glutamate is a potent neurotoxin has long been recognized. It is responsible for the toxic death of postsynaptic neurons. Both focal and global cerebral ischemia increase extracellular glutamate levels [23-25].

Prolonged and excessive stimulation of glutamatergic receptors occurs when extracellular glutamate approaches 100 pM [26]. Even at normal concentrations, glutamate can be neurotoxic in energetically disadvantaged cells. This dominant excitotoxic action occurs as a consequence of strong or prolonged activation on postsynaptic glutamatergic receptors. Increasing the amount of glutamate causes the hyperstimulation of neurons with increasing influx of Na^+ and Ca^{2+}. The water passively follows the influx of ions resulting in cytotoxic neuronal edema [27-29].

The activation of glutamate receptors causes an influx of calcium from the extracellular environment into the intracellular compartment, leading to calcium overload of the mitochondria and the activation of calcium-dependent catabolic enzymes. Glutamate, in high concentration, causes primary neuronal necrosis. In spite of this however, using pharmacological inhibitors of glutamate receptors develops an apoptotic lesion mechanism that may predominate under certain pathophysiological conditions. The importance of excitotoxicity for ischemic cell lesions has been intensely debated, but this has not offset the beneficial effect of glutamate antagonists in the treatment of focal ischemia [30,31].

Calcium toxicity

In the intact cell, highly efficient calcium transport systems ensure that an extremely large difference in calcium concentration of approximately 1:10,000 is

maintained between the extracellular and intracellular compartment on the one hand, and between the cytosol and the endoplasmic reticulum (ER) on the other. During anoxic ischemic depolarization, there is a sudden increase in the amount of cytosolic calcium in association with the activation of glutamate receptors [32]. At the onset of ischemia, the increase in calcium in the cytosol is further enhanced by the metabolic activation of glutamate receptors that mediate the release of calcium from the endoplasmic reticulum (ER) [33].

The prolonged high calcium concentration in the cytosol causes mitochondrial dysfunctions and induces catabolic changes, especially by activating Ca^{2+} -dependent effectors of protein and enzyme types such as endonucleases, phospholipases, protein kinases and other proteases that damage DNA, lipids and proteins. The release of calcium from the endoplasmic reticulum evokes a stress response to it, which mediates a large number of endoplasmic reticulum-dependent secondary disorders, especially the inhibition of protein synthesis. Calcium-dependent pathological events are therefore complex and contribute to a multitude of secondary molecular lesions [34,35].

Intracellular Ca^{2+} growth appears to play a major role in pathological events following excitotoxicity [36]. The activation of N-methyl D-aspartate (NMDAR) receptors is the main source of intracellular Ca^{2+} in normal and ischemic cells [37-39].

Mitochondrial dysfunction is another consequence of the increase in intracellular Ca^{2+} concentration.

Increasing intracellular Ca^{2+} above a certain threshold (approximately 0.5 μM) will increase its absorption in mitochondria [40]. Ca^{2+} content in the mitochondria increases from 1-3 nmol/mg protein to 6-9 nmol/mg protein after 24 hours of reperfusion [41-44].

This increase causes devastating effects, including the transitional opening of mitochondrial pore permeability, complete loss of cellular respiration, and the release of both NADH and cytochrome c [45,46].

Impairment of the mitochondrial function and mitochondrial collapse lead to the inability to maintain membrane potential, antioxidant mechanisms and the generation of oxygen free radicals (ROS), and inhibition of intracellular Ca^{2+} growth will inhibit this cascade [47-49]. Loss of NADH in the mitochondria occurs as a result of transitional pore permeability opening (MPTP).

The role of free radicals (ROS/RNS) and antioxidants in neuronal ischemia

Glutamate hyperactivity is associated with the production of various reactive oxygen species (ROS). These can cause damage by oxidizing lipids, DNA and proteins. Ca^{2+} accumulation induces the activation of calpains, neutral proteases, which leads to the conversion of xanthine dehydrogenase to xanthine oxidase. Xanthine oxidase catalyses the oxidation of xanthine and hypoxanthine to uric acid, which produce a superoxide [50]. Excess Ca^{2+} disrupts the electron transport chain in the mitochondria, they accumulate in the mitochondria and react with oxygen, which is supplied after reperfusion causing superoxide production. The superoxide is further processed to produce hydroxyl radicals by a Fenton reaction or peroxynitrite by reaction with nitrogen oxide. ROS also inhibit the electron transport chain in mitochondria [51,52].

Free radicals

In regions of the brain with a low level of infusion or intermittent blood infusion, reactive oxygen species (ROS) that cause peroxidative damage to plasma membranes and intracellular organs are formed [53,54]. Reacting with nitric oxide leads to the formation of peroxynitrates, which also cause violent biochemical reactions. Biologically, the side effects of free radical reactions are the release of active free fatty acids, such as arachidonic acid, the induction of stress in the endoplasmic reticulum, the induction of mitochondrial disorders and DNA fragmentation.

The latter can induce apoptosis and thus increase molecular lesions [55,56].

Many studies have shown that reactive oxygen radicals play an important role in the pathophysiology of various neurological diseases [57,58]. Experimental ischemia and reperfusion patterns, such as focal/transient ischemia in rodents, suggest the involvement of oxygen radicals in the pathogenesis of ischemic lesions. In these models, cerebral blood flow is reduced by the occlusion of cerebral vessels. Re-oxygenation during reperfusion provides a substrate for numerous enzymatic oxidation reactions. Mitochondria produce anionic superoxide and hydrogen peroxide (H_2O_2) radicals under normal physiological conditions. These products are constantly converted to reactive oxygen species (ROS) of superoxide dismutase (SOD), glutathione peroxidase ($GSHP_x$) and catalase. SOD specifically processes superoxide anion (O_2) and produces H_2O_2, which is then detoxified by catalase or GSHPx and eventually converted to water and superoxide. Hydroxyl radicals can be generated from H_2O_2 by the Fenton reaction ($H_2O_2 + Fe^{2+} = OH + Fe^{3+} + OH$) [59,60].

Other small molecule antioxidants including glutathione, ascorbic acid, α-tocopherol, are involved in free radical detoxification. Reperfusion after ischemia causes overproduction of ROS in the mitochondria and consumption of endogenous antioxidants by these radicals can lead to a dramatic increase in intracellular ROS. ROS have been shown in numerous studies to be directly associated with cellular macromolecules such as lipids, proteins, and nucleic acids in the oxidative damage of nucleic acids in ischemic tissue leading to

cell death. Recent studies have shown that indirect ROS-mediated signalling pathways can cause cell injury and death in cerebral ischemia and reperfusion [61-65].

Superoxide dismutases

Superoxide dismutases (SODs) are specific antioxidant enzymes that detoxify O_2 and produce H_2O_2. There are three SODs namely, copper/zinc SOD (SOD1), manganese SOD (SOD2) and extracellular SOD (ECSOD). These are major antioxidant enzymes based on cell distribution and localization.

SOD1 is a major cytosolic enzyme, accounting for about 0.1% of the total protein in mammalian cells. SOD2 is a mitochondrial enzyme, and ECSOD is an isoform enzyme located in the extracellular space, cerebrospinal fluid, and cerebral blood vessels [66]. All three SOD isoforms convert O_2, forming H_2O_2 which is removed by catalysis or GSHPx based on GSH consumption. GSH is generated from the oxidation of GSH by GSH-reductase in the reduced presence of dinucleotide-adenine-nicotinamide phosphate. Other lipid peroxides are also removed by GSHPx. SOD1 has been used extensively in experimental studies involving cerebral ischemia and reperfusion [67-73].

In global ischemia, overexpression of SOD1 is neuroprotective, with a 50% reduction in CA1 hippocampal cell death [74,75] and this protection is probably partly due to the blockage of the apoptosis' mitochondrial pathway [76,77].

The role of SOD1 in cerebral ischemia is further confirmed by the use of SOD-deficient mice. These SOD1-deficient mice had increased cell death and edema after cerebral ischemia through transient and global median cerebral artery occlusions [78].

The importance of mitochondrial oxygen radical production and the protective role of SOD2 after permanent cerebral ischemia has been demonstrated in SOD2-deficient mice. These mutant mice showed extensive infarction after permanent cerebral artery ischemia and increased release of mitochondrial cytochrome c and subsequent DNA fragmentation after permanent focal cerebral ischemia [79,80]. However, mice overexpressing SOD2 showed neural protection against oxidative stress after transient focal cerebral ischemia. The level of ECSOD in the brain is much lower than in other organs, but recent studies have shown that overexpression of this protein provides protection after focal and global ischemia, as sacrificed animals showed a greater infarction after focal ischemia [81-83].

Nitric oxide toxicity

Nitric oxide (NO) is a product of NO-synthetase (NOS), which acts on arginine.

There are at least three NOS isoforms, namely eNOS (expressed in endothelial cells), nNOS (in inducible neurons and isoenzymes) and iNOS (mainly in macrophages).

Pathophysiologically, NO has two opposite effects: in endothelial cells the generation of NO leads to vasodilation, an improvement in blood flow and reduction of hypoxic lesions, while in neurons it contributes to the excitotoxicity of glutamate and, through the formation of peroxynitrate, to the apparition of free radical damage [84,85].

The inhibition of protein synthesis is a significant molecular marker for assessing the progression of ischemic lesions. The inhibition of protein synthesis persists throughout the whole period from the onset of ischemia to cell death [86].

This process is initiated by ischemia-induced calcium release from the endoplasmic reticulum (ER), leading to endoplasmic reticulum stress [87-89]. In addition, the activation of protein kinase R (PKR) results in the disaggregation of ribosomes and the inhibition of protein synthesis.

CONCLUSIONS

The molecular mechanisms involved in cellular and tissue changes caused by ischemia are extremely complex and incompletely deciphered. Our study data show that shortly after the onset of ischemia, a number of excitatory and inhibitory neurotransmitters are released, resulting in the activation of their specific receptors. Among these neurotransmitters, special attention is given to glutamate, which at high concentrations is known to produce excitotoxicity. The activation of inotropic glutamate receptors causes an influx of calcium from the extracellular environment into the intracellular compartment, which leads to calcium overload of mitochondria and the activation of calcium-dependent catabolic enzymes.

In high concentrations, glutamate causes primary neuronal necrosis.

Also, during anoxic ischemic depolarization, there is a sudden increase in the amount of cytosolic calcium. At the onset of ischemia, the increase in calcium in the cytosol is further enhanced by the metabolic activation of glutamate receptors that mediate the release of calcium from the endoplasmic reticulum. The changes in the concentration of intracellular calcium are extremely pathogenic, an aspect highlighted in the present study.

Our study also shows that in regions of the brain with a low level of infusion or intermittent infusion of blood, reactive oxygen species are formed that cause peroxidative damage to plasma membranes and intracellular organs. Biologically, the side consequences of free radical reactions are the release of active free fatty acids, such as arachidonic acid, the induction of stress

in the endoplasmic reticulum, the induction of mitochondrial disorders and DNA fragmentation. The latter can induce apoptosis and thus aggravate molecular lesions. Nitric oxide is a product of NO-synthetase (NOS), which acts on arginine. Physiologically, nitric oxide has two opposite effects, namely in the endothelial cells the generation of nitric oxide leads to vasodilation, an improvement in blood flow and reduction of hypoxic lesions, while in neurons it contributes to the excitotoxicity of glutamate and, through the formation of peroxynitrate, to the apparition of lesions determined by free radicals.

A significant molecular marker for the progression of ischemic lesions is the inhibition of protein synthesis, which persists throughout the whole interval from the onset of ischemia to cell death. It is initiated by the release of calcium induced by endoplasmic reticulum (ER), which leads to ER stress and abnormalities in various biological cells, such as the appearance of abnormal proteins, stress protein expression and a global inhibition of protein synthesis, the latter also due to the activation of protein kinase R, which causes the disaggregation of ribosomes and the inhibition of protein synthesis during transduction.

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