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Review

Molecular pathophysiological mechanisms of ischemia/reperfusion injuries after recanalization therapy for acute ischemic stroke

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With the larger variety of methods employed, recanalization therapy is increasingly used to treat acute ischemic stroke resulting in about one-third of patients undergoing early neurological deterioration, in which ischemia/reperfusion injuries are the main cause, leading to increases in the infarcted area, the no-reflow phenomenon, or hemorrhagic transformation. Efficient prevention or treatment of these injuries depends on extensive knowledge of the involved mechanisms. These pathways have dual, damaging, and neuroprotective effects, depending on the timing or protein subtype involved. The current article reviews the main mechanisms contributing to the pathophysiology of these injuries, such as mitochondrial dysfunction, cellular calcium overload, excitotoxicity, oxidative stress, apoptosis, and neuroinflammation.

Keywords

Reperfusion injury; Excitotoxicity; Mitochondria; Oxidative stress; Apoptosis; Neuroinflammation

1. Introduction

The concept of ischemia/reperfusion injury emerged over 50 years ago when Jennings and coworkers showed that in hearts subjected to coronary ligation, reperfusion accelerated the development of necrosis [1]. Paradoxically, restoring blood supply to an organ subjected to temporary glucose and oxygen deprivation can injure the tissue [2, 3], as described in the kidneys, intestines, skeletal muscles, liver, and cerebral tissue [4]. Cellular and molecular mechanisms contribute to ischemia/reperfusion injuries, involving reactive oxygen species (ROS), innate and adaptive immune systems, and dysfunction of cellular metabolism and vascular and parenchymal cellular demise [5, 6]. Although much of the research has been performed in animal models, with abrupt reperfusion after transient ischemia [7] increasing the size of the infarction by as much as 70% [8], in human patients, the increase in infarct size in the first 24 hours is more limited [9]. However, hyperperfusion (defined as $\geq$100% increase in cerebral blood flow compared with baseline) [10] or normalization of blood flow (reperfusion) can significantly potentiate the magnitude of the tissular damage inflicted by the initial ischemic insult and manifest clinically as headache, worsening of the neurological deficit, seizures, or histologically as cerebral edema, hemorrhagic transformation, extension of the infarct size [11], with a delayed cellular loss which can extend up to 2 weeks after the initial ischemic event [12].

A large amount of research has focused on unraveling the complex mechanisms of ischemia/reperfusion (I/R) injuries which are caused by a complex interplay between mitochondrial dysfunction, oxidative and nitrosative stress, calcium overload and excitotoxicity, activation of apoptosis, and inflammation [4]. This knowledge can open novel therapeutic opportunities for preventing them and extend the therapeutic windows for recanalization procedures.

2. Mitochondrial dysfunction

Mitochondria are intracellular organelles with a double membrane that have a crucial role in energy generation, regulation of cell cycle, and apoptosis induction [13]. The inner membrane contains a series of enzyme complexes responsible for oxidative phosphorylation (complexes I–V) and the generation of adenosine triphosphate (ATP) [14].

Complex I or proton-pumping nicotinamide adenine dinucleotide (NAD) H dehydrogenase oxidizes NADH by pumping 4 protons per 2 electrons passed to ubiquinone, resulting in ubiquinol (QH_2) [14, 15] and is the main access point for electrons. Further, complex II, or succinate-quinone oxidoreductase, is a second entry point of electrons into the electron transport chain, which oxidizes succinate to fumarate and reduces ubiquinone. Complex III, or cytochrome c reductase, oxidizes one molecule of ubiquinol by reducing 2 molecules of cytochrome c, also pumping 2 protons. Cytochrome c oxidase, or complex IV, reduces oxygen to water by transferring electrons to oxygen from the reduced cytochrome c, pumping an additional 4 protons. Finally, ATP synthase, or complex V, synthesizes ATP from ADP and phosphate by using the energy of the proton electrochemical gradient, a reaction during which 4 protons re-enter the matrix [13, 14]. Under normal conditions, more than 90% of oxygen is reduced to water and approximately 2% of electrons "leak" mainly from complexes I and III to produce superoxide anion [13, 16].

The lack of oxygen during ischemia inhibits the electron flow through the respiratory chain, preventing ATP synthase from generating ATP [17]. The rate of entry of electrons into complex I exceed the rate of transit through complex IV, causing them to build up at complexes I and III and slowing down the electron transport chain and the pumping of protons across the inner mitochondrial membrane, leading to a reduction of the mitochondrial membrane potential [16, 18].

Following the restoration of blood flow, the mitochondrial membrane potential is restored within 1 minute [19]. Still, the increased oxidative phosphorylation leads to mitochondrial hyperpolarization with dramatic consequences on the mitochondrial function and the increased generation of reactive oxygen species (ROS), which will further impair the normal mitochondrial function [20]. Indeed, after 30 minutes following reperfusion, mitochondrial function is significantly decreased in cells that will die [21].

Mitochondria are organelles whose dynamics, regulated by fission and fusion, have an important role in neuronal injury and recovery following ischemia [14]. Fission manifested as constriction and cleavage of mitochondria is regulated by dynamin-related protein 1 (Drp1), a mitochondrial-binding GTPase. It has been shown that global cerebral ischemia transiently increases phosphorylation of Drp1 [22] while Drp1 inhibitors reduced the infarct volume in a model of focal cerebral ischemia [23]. Mitochondrial fission also can initiate extrinsic apoptotic cell death, and fragmentation of these organelles in endothelial cells leads to endothelial dysfunction in postischemic tissues [4, 24].

3. Calcium overload and excitotoxicity

During ischemia-hypoxia, the brain cells switch to anaerobic glycolysis to supply the necessary ATP, which leads to the accumulation of lactate, NAD^+ and protons. Trying to re-establish normal intracellular pH, the plasmalemmal Na^+/H^+ exchanger (NHE) extrudes protons in exchange for Na^+ [25], followed by an exchange of Na^+ for Ca^{2+} mediated by the Na^+/Ca^{2+} exchanger [4]. This leads to a calcium overload of the cell. Normal cytosolic free calcium concentrations are in nanomolar ranges instead of minimolar levels in the extracellular space [26]. In addition, the release of excitatory neuromediators, mainly glutamate, because of cellular depolarization or destruction, further exacerbates this calcium overload in neighboring and distant sites. By removing extracellular H^+ ions, Reperfusion accelerates the activity of the NHE and increases intracellular calcium levels [4, 27].

High intracellular calcium promotes calcium from the endoplasmic reticulum via activated ryanodine receptors [28]. It proves toxic by activating a series of enzymes, such as the family of cysteine proteases known as calpains, which degrade cytoskeletal, mitochondrial proteins and the endoplasmic reticulum [29]. Research has shown that pharmacological inhibition of calpains can protect the brain against reperfusion injuries [30]. Another important pathway triggered by increased cytosolic calcium levels is the activation of Ca^{2+}/calmodulin-dependent protein kinases (CAMKs), which translocate to the synaptosomes, phosphorylate N-methyl-D-aspartate receptors (NMDARs) and α-amino-3-hydroxy-5-methylisoxazole-propionic acid receptors (AMPARs), thereby further increasing Ca^{2+} influx, and phosphorylate Beclin-1 inducing autophagy [31].

High intracellular calcium levels also lead to the generation of danger signals, such as calcium pyrophosphate complexes and uric acid, which bind to the inflammasomes (intracellular protein complexes) and lead to the increased production of cytokines initiating inflammation [4].

Mitochondria act as a calcium buffer, attempting to normalize the cytosolic calcium levels. The ion moves through the outer mitochondrial membrane through the voltage-dependent anion-selective calcium channel and further into the mitochondrial matrix mediated by the mitochondrial calcium uniporter [32, 33]. However, excessive mitochondrial Ca^{2+} further impairs mitochondrial function and can trigger the mitochondrial permeability transition pore [34].

As already mentioned, excess excitatory neuromediator (glutamate) release significantly increases cellular calcium overload. Glutamate binds mainly to 2 ionotropic, ligand-gated ion channels: NMDARs and AMPARs. In the resting state, magnesium blocks the channel pores of NMDARs. Glutamate binding to AMPARs causes a partial depolarization of the postsynaptic membrane, which removes Mg^{2+} and allows the NMDARs to be activated with a subsequent entry of Na^+ and Ca^{2+} into the cell [35, 36]. There are several subtypes of NMDARs, heterotetramers consisting of 2 GluN1 subunits and 2 GluN2 subunits, which can be further classified in GluN2A-GluN2D [37]. NMDARs are essential for brain development, synaptic plasticity, and learning, but they can initiate toxic pathways that lead to neuronal death when excessively activated. It appears that NMDARs have dual roles in neuronal survival and death depending on the location and subtype of receptor-activated [38]. Synaptic NMDARs are mainly GluN2A receptors, while extrasynaptic ones contain mainly the GluN2B subunit [39]. Stimulation of the synaptic NMDARs activates phosphoinositide-3-kinase (PI3K), phosphorylating membrane phospholipids and Akt [40]. Akt, in turn, phosphorylates and inactivates glycogen synthase kinase 3β (GSK3β), pro-apoptotic Bcl-2 associated death promotor BAD, JNK (c-Jun N-terminal kinase)/p38 activator ASK1 (apoptosis signal-regulating kinase 1), and apoptotic p53 [41–43]. In addition, synaptic NMDAR stimulation activates the Ras/ERK (extracellular signal-regulated kinase) pathway and nuclear CAMKs, which activate CREB (cAMP-response element binding protein) and

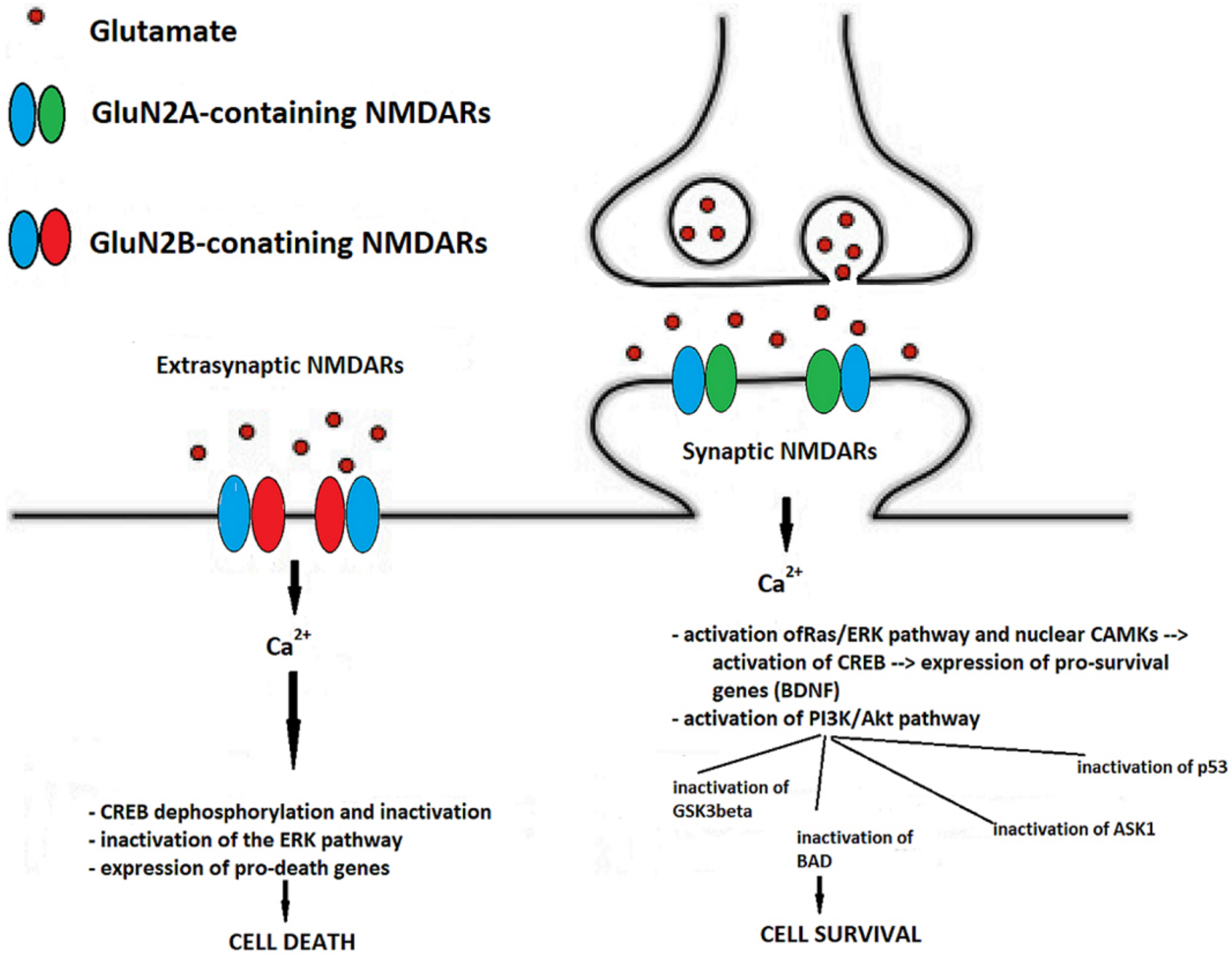

Fig. 1. The dual role of NMDA receptors in determining the fate of neurons: binding of glutamate to extrasynaptic NMDARs dephosphorylates cAMP-responsive element-binding protein (CREB), inactivates the extracellular signal-regulated kinase (ERK) pathway and promotes cell death, while binding of glutamate to synaptic NMDARs promotes cell survival through activation of the phosphoinositide-3-kinase (PI3K)/Akt pathway, which inactivates glycogen synthase kinase 3β (GSK3β), the pro-apoptotic Bcl-2 associated death promotor (BAD), pro-apoptotic p53, and c-Jun N-terminal kinase (JNK)/p38 activator apoptosis signal-regulating kinase 1 (ASK1). Adapted from [35].

induce pro-survival gene expression, such as brain-derived neurotrophic factor (BDNF) [44, 45]. Thus, the binding of glutamate to synaptic NMDARs promotes cell survival. The binding of glutamate to extrasynaptic NMDARs dephosphorylates and inactivates CREB, inactivates the ERK pathway and promotes pro-death gene expression [46, 47].

Under physiological conditions, presynaptic axonal terminals release quanta of glutamate into the synaptic cleft to activate receptors on the postsynaptic membrane [48]. Astrocytes clear glutamate from the synaptic cleft through specific transporters (excitatory amino acid transporters—EAATs) and transform it into glutamine or use it for their metabolism, thereby maintaining glutamate homeostasis [49, 50]. However, this is a highly energy-consuming process, which fails in oxygen and glucose deprivation conditions, as happens in ischemic conditions. Glutamate uptake via EAATs occurs with co-transport of $3Na^+$ and $1H^+$, followed by the counter-transport of K^+, making glutamate uptake possible against a significant concentration gradient [49]. The impaired energy supply caused by ischemia leads to decreased activity of the Na^+/K^+ ATPase and disruption of the Na^+ and K^+ transmembrane gradients, impairing the capacity of the EAATs to clear glutamate and leading to increased extracellular glutamate concentrations, which can diffuse onto the myelin sheath and be trapped in the periaxonal space between the internal myelin surface and the axolemma [51], thereby creating the premise for glutamate to act on extrasynaptic receptors and initiate the deleterious downstream effects discussed above. Fig. 1 (Ref. [35]) shows the dual roles of the 2 types of NMDARs.

4. Oxidative stress

Reperfusion of ischemic tissue with oxygenated blood, although necessary for aerobic ATP production, leads to increased production of ROS, which can oxidize almost every biomolecule and induce cell dysfunction (oxygen paradox)

[4]. Oxidative stress, defined as an imbalance between ROS production and the ability of the biological system to clear these highly reactive molecules, has been shown to significantly contribute to the pathophysiology of I/R injuries [52].

Three distinct phases of increased ROS generation have been identified in cell cultures [18, 53]: (i) during glucose and oxygen deprivation, due to mitochondrial depolarization and inhibition of complex IV leading to upstream accumulation of reduced compounds which enable leakage of electrons; (ii) 25–35 minutes after the oxygen and glucose deprivation, caused by activation of xanthine oxidase; (iii) after reperfusion.

The brain is particularly vulnerable to oxidative stress due to a series of characteristics: (i) it has the highest metabolic activity per unit weight, consuming 20–25% of the total body oxygen despite weighing only 2% of the total body weight [6, 13]; (ii) compared to other organs, such as the heart, kidney, or liver, it has significantly lower activities of antioxidants such as superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase, or heme-oxygenase-1 [54, 55]; (iii) it has lower activities of Cytochrome c oxidase, resulting in higher superoxide release from the mitochondrial electron transport chain during ATP production, which is also reduced [54]; (iv) the plasma membranes of brain cells are rich in polyunsaturated fatty acids, highly vulnerable to oxidative damage [56]; (v) it has a high ratio of membrane surface area compared to the cytoplasmic volume [57]; (vi) damaged cerebral parenchyma releases iron ions which can catalyze free radical reactions [57]; (vii) excessive neurotransmitter release during ischemia/reperfusion, such as glutamate and dopamine, resulting in cellular calcium overload, which impairs mitochondrial function and leads to excitotoxicity [4, 6].

The main ROS include superoxide anion (O_2^-), hydroxyl radicals (OH^-), and hydrogen peroxide (H_2O_2) [58]. The main sources of reactive species are the mitochondria, the activity of cyclooxygenases, NADPH oxidase (NOX), lipoxygenases and other enzymes, and the activation of xanthine oxidase [59, 60].

4.1 Sources of ROS

4.1.1 Mitochondria and oxidative stress during reperfusion

Cerebral ischemia inhibits the activity of complex I, leading to the accumulation of succinate through the reversed activity of succinate dehydrogenase, which reduces fumarate to succinate, and to a lesser extent, the activity of complex IV (cytochrome C oxidase) [61, 62]. The reduced activity of the final electron acceptor in the mitochondrial electron transport chain causes increased ROS production of upstream complexes, dramatically increased after oxygen delivery is restored by reperfusion [62, 63]. In addition, upon reperfusion, succinate dehydrogenase oxidizes the accumulated succinate and drives reverse electron transport through complex I, which is why complex I is regarded as the main ROS-generating mitochondrial site [61, 64]. However, at least 7 sites in the mitochondria can partially reduce oxygen and produce ROS [65, 66].

One class of enzymes mitigating the effects of ROS are the superoxide dismutases (SODs), with manganese SOD (MnSOD) being mainly a mitochondrial enzyme and copper-zinc SOD (Cu-ZnSOD) a cytosolic one. Complex I dysfunction after reperfusion influences MnSOD expression [62].

Reperfusion is associated with large increases in intracellular and mitochondrial Ca^{2+}, leading to mitochondrial depolarization. In this situation, calcium exits the mitochondria by forming pores in the mitochondrial membrane, reversing the Ca^{2+}/H^+ antiport system or through channel-mediated pathways [62]. Increased cytosolic calcium triggers apoptosis through activation of a series of proteases, phospholipases, and nucleases.

The outer mitochondrial membrane is associated with 2 monoamine-oxidases, monoamine oxidase-A and -B, which deaminate neurotransmitters at the expense of generating hydrogen peroxide [67].

Another highly reactive molecule produced by mitochondrial is nitric oxide (NO), which, at physiological concentrations, reversibly inhibits Cytochrome c oxidase and modulates oxygen consumption [68]. More recently, research has shown the involvement of another protein, p66Shc, located between the 2 mitochondrial membranes and forms molecular complexes with cytochrome c, thereby transferring electrons between itself and cytochrome c. It appears that this protein also contributes to increased ROS production, mitochondrial depolarization, and cytochrome c release [69, 70].

The mitochondrial permeability transition pore (MPTP) is a key player in I/R injury. It is inhibited by low pH, so it is quiescent during ischemia, but the increases in mitochondrial Ca^{2+} and cellular ROS levels associated with reperfusion lead to the opening of the MPTP [62, 71]. This allows protons to pass into the matrix and dissipate the mitochondrial membrane potential, allows water to enter mitochondria through the osmotic gradient leading to swelling and even rupture of the organelles, and is an essential step in initiating apoptosis [72, 73]. Fig. 2 (Ref. [74]) presents the implication of mitochondria in cerebral ischemia/reperfusion injuries schematically.

4.1.2 NADPH oxidase as a source of ROS

NADPH oxidase (NOX) is a membrane enzymatic complex that generates superoxide while transferring electrons from NADPH to oxygen molecules across the cell membrane [59]. However, NOX is the main defense mechanism of macrophages and neutrophils. Exposure to microorganisms or inflammatory mediators can increase 50- to 100-fold the production of oxidative species [4]; NOX2 and NOX4 isoforms have been localized in the hippocampal CA1 region and cortex [68]. Experimentally, NOX2 knockout animals and NOX2 inhibitor-treated animals showed significantly reduced infarct sizes, demonstrating the role of NOX2 in oxidative stress-induced ischemic neuronal death [75]. The vascular NOX isoforms usually have lower activity levels, the ROS generated by them being more likely involved in signal-

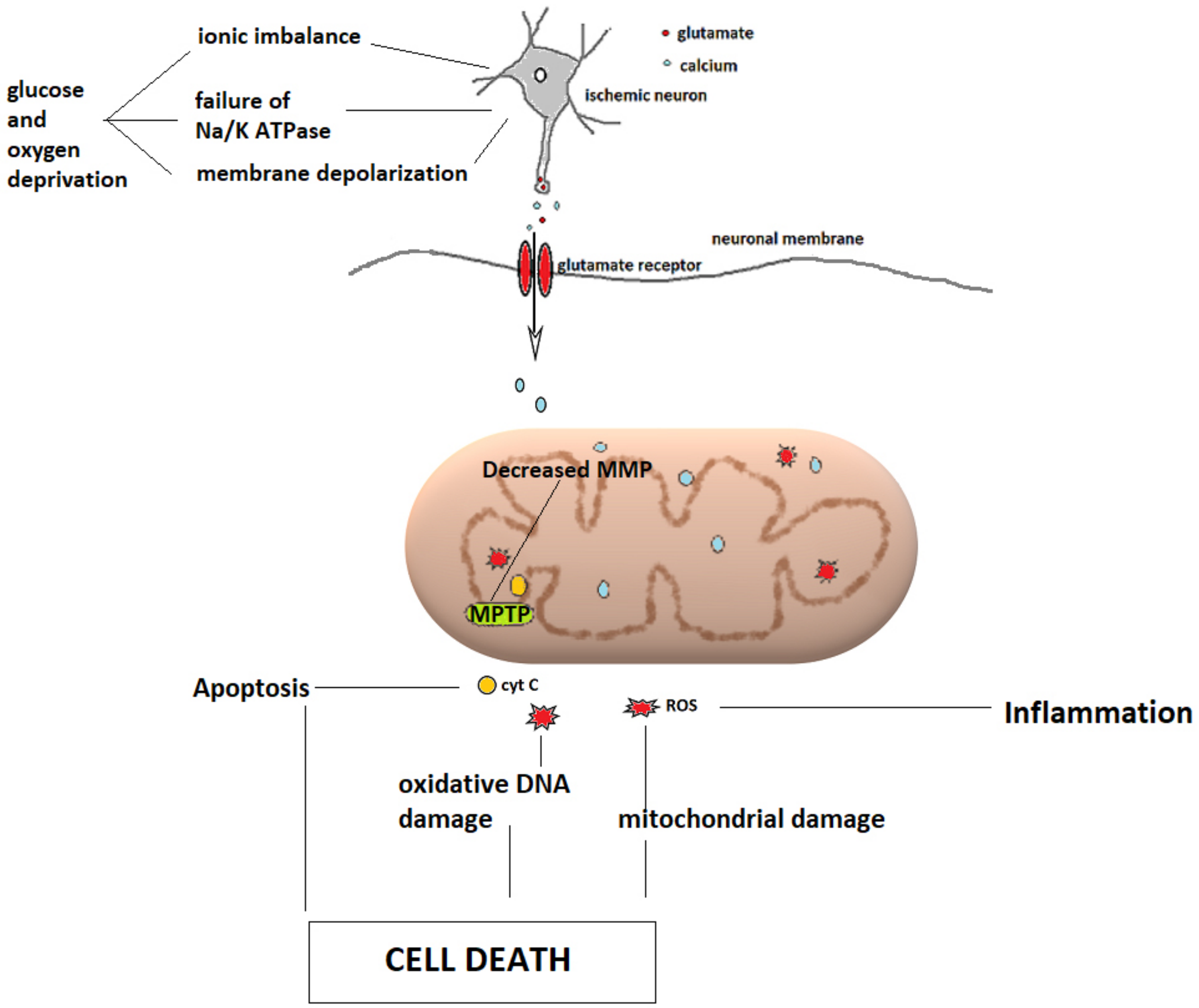

Fig. 2. During ischemia, oxygen and glucose deprivation leads to failure of Na^+/K^+ ATPase pump, which results in neuronal membrane depolarization and excessive releases of glutamate, which activates glutamate receptors and leads to excessive calcium influx. Excessive intracellular Ca^{2+} leads to ROS production and mitochondrial dysfunction, depolarization of mitochondrial membrane potential (MMP), and opening of the mitochondrial permeability transition pore (MPTP), with the release of cytochrome C (cyt C), which triggers apoptosis. Excessive ROS further damages mitochondria and nuclear DNA, leading to cell death and contributing to inflammation initiation. Adapted from [74].

ing cascades. However, after ischemia-reperfusion, vascular NOXs can produce increased levels of ROS and produce oxidative stress [76].

4.1.3 Xanthine oxidase as a source of ROS

Xanthine oxidase (XO) is a molybdo-flavin enzyme that exists in 2 forms: a NAD-dependent dehydrogenase (xanthine dehydrogenase) and an oxygen-dependent oxidase (xanthine oxidase) with a higher affinity for oxygen than NAD^+ and which produces hydrogen peroxide [77]. During reperfusion, xanthine dehydrogenase is converted by oxidation or limited proteolysis to xanthine oxidase, activated by phosphorylation and produces ROS [78]. Thus, under hypoxic conditions, xanthine oxidase metabolizes hypoxanthine and xanthine, generating oxidative species [18]. Experimentally, inhibiting XO results in less calcium overload, diminished levels of markers of oxidative stress, reduced magnitude of tissue injury [52], and reduced leucocyte recruitment and accumulation, leading to diminished levels of inflammation [79].

4.1.4 Nitric oxide synthases

The central nervous system expresses 3 kinds of nitric oxide synthases (NOS): endothelial NOS (eNOS), which regulates cerebral blood flow, neuronal NOS (nNOS), and inducible NOS (iNOS). Nitric oxide (NO) produced by eNOS after brain ischemia promotes vasodilation and inhibits microvascular adhesion and aggregation, thus exerting a protective effect [68]. However, ischemia activates nNOS through the high intracellular Ca^{2+} levels and upregulates iNOS in an NF-κB-dependent manner, both of which significantly contribute to brain damage [68]. Experiments with nNOS knockout mice and with NO inhibitors showed reduced infarct volumes after ischemia [80, 81]. Nanomolar concentrations of NO can reversibly inhibit cytochrome C oxidase, while higher levels can irreversibly modify proteins, lipids and impair mitochondrial respiration [62]. By reacting with O_2^-, NO leads to the formation of peroxynitrite ($ONOO^-$) [82, 83], which diffuses through mitochondrial compartments alter proteins of the matrix, intermembrane space, as well as of the outer and inner membrane, and impair the mitochondrial calcium and energy homeostasis leading to the opening of the permeability transition pore [84].

4.1.5 Other sources of ROS

ROS can also result from the activity of other intracellular enzymes, such as cytochrome P450 enzymes, cyclooxygenases, or lipoxygenases [59].

Cytochrome P450 enzymes (CYPs) are membrane-bound oxidases that use oxygen or NADPH to catalyze oxidation or reduction of lipids, steroids, cholesterol or other lipids, such as arachidonic acid [4]. They have a crucial role in vasoregulation, forming both vasoconstrictive compounds, such as 20-hydroxyeicosatetraenoic acid (20-HETE) and vasodilator epoxyeicosatrienoic acids [85]. The role of CYPs in I/R injury is complex, but research has suggested that 20-HETE may be significantly involved in the pathophysiology of these injuries, at least in neonatal brains [86]. Moreover, cerebral ischemia induces CYP expression [87].

Lipoxygenases (LOXs) catalyzes the synthesis of eicosanoids, such as leucotrienes and hydroxyeicosatetraenoic acids. Following cerebral ischemia, there is a massive release of free fatty acids from membrane stores [88]. 12/15 LOX oxidizes these lipids, leading to the generation of 12- and 15-HETE [89], and can damage the mitochondrial membrane, leading to increased ROS production and initiating apoptosis [90]. Experimentally, inhibition of 12/15 LOX with baicalein resulted in reduced infarct volume, similar to infarctions of animals in which ALOX15, the gene encoding for LOX12/15, was knocked out [91].

Another key enzyme in the generation of prostaglandins from arachidonic acid is cyclooxygenase (COX) [92, 93]. Both COX-1 and COX-2 isoforms cleave arachidonic acid, and upregulation of COX-2 is a hallmark of ischemia/reperfusion injuries [94], especially in the inflammatory cells, which invade the cerebral tissue after an ischemic injury [95]. Pharmacological inhibition or genetic inactivation of COX-2 resulted in the reduced magnitude of cerebral injury after focal or global cerebral ischemia [96, 97], although COX's radical species have never been identified [98]. The reports on increased incidence of cardiovascular events, including stroke, after long-term treatment with COX-2 inhibitors, have challenged these agents' therapeutic potential [98, 99].

4.2 Antioxidant defenses

Under normal conditions, the small amounts of ROS can be removed by the brain's antioxidant enzymatic and non-enzymatic defenses. The antioxidant enzymes include superoxide dismutase (SOD), glutathione peroxidase (GPX), and catalase (CAT) [59]. Non-enzymatic antioxidant molecules are present mainly in extracellular spaces and include glutathione, vitamins C and A, N-acetylcysteine and melatonin [59, 100]. However, following cerebral ischemia, and especially after reperfusion, the production of ROS increases [93, 101], which, together with the downregulation of the enzymatic antioxidant defenses by ischemia [102], leads to significantly increased oxidative stress and oxidative species-induced cellular injury.

4.3 Effects of reactive oxygen species in ischemic stroke

ROS has a series of detrimental effects, initiating several cell signaling cascades and altering the functions of enzymes and ion channels.

ROS can activate p53, a transcription factor controlling the gene expression of Bax, Bid and p53 upregulated modulator of apoptosis (PUMA). P53 opens the mitochondrial permeability transition pore and increases the mitochondrial membrane permeability (also caused by ROS), leading to cytochrome c release [60, 103]. This is pivotal in initiating apoptosis because released cytochrome c forms a complex with apoptotic protease activating factor-1 (APAF-1), pro-caspase-9 and ATP, and activates caspases [104]. In addition, p53 upregulates apoptosis signal-regulating kinase 1 (ASK1), which together with PUMA is involved in executing apoptotic cell death [105, 106].

Mitogen-activated protein kinases (MAPKs) are a family of serine/threonine kinases with substantial cell growth, survival, proliferation, and death. The 3 main MAPKs are extracellular signal-regulated kinases (ERKs), c-Jun N-terminal kinases (JNKs), and the p38 MAPKs [4]. ERKs are protective against ischemia-reperfusion injuries [4], the role of JNKs is controversial [107, 108]. At the same time, p38 MAPK is activated in response to I/R [109] but, depending on the isoform activated, can be either protective or harmful: it appears that activation of p38A is lethal to the cell [110]. In contrast, activation of the B isoform of p38 is cytoprotective and involved mainly in preconditioning [111].

ROS can interact with a variety of biological molecules. They can react with proteins, leading to their oxidation, degradation, or peptide bond cleavage, resulting in protein aggregation, enzyme inactivation, or modifications in the activity of ion channels [112, 113]. For example, oxidation and inactivation of glutamine synthetase in astrocytes prevent glutamate's conversion into glutamine and contribute to ischemia-induced neurotoxicity in the gerbil brain [114].

Lipid peroxidation (ROS attacking the carbon-carbon bonds of polyunsaturated fatty acids) is even more damaging than protein oxidation, being self-propagated because lipid radicals are unstable and react with oxygen to form lipid peroxyl radicals [59, 115]. These can react with other fatty acids to generate aldehydes, such as malondialdehyde and 4-hydroxynonenal, the latter being a second messenger which regulates several transcription factors including NF-κB, activating protein1, nuclear factor erythroid 2-related factor 2, or peroxisome-proliferator-activated receptors (PPARs), as well as the phosphatidylinositol 3kinase (PI3K)/protein kinase B (Akt) signaling pathway involved in cell cycle, cell growth and proliferation [59, 116].

Finally, ROS can attack the DNA causing double-strand breaks, protein-DNA crosslinks, structural changes, or DNA mutations [117, 118], leading to increased poly (ADP-ribose) polymerase (PARP) activity in an attempt to repair DNA damage but which depletes the cells of the already reduced energy supplies [119].

5. Apoptosis

This mechanism of cell death, with distinct features from necrosis, can be initiated by 2 main pathways: the extrinsic pathway, related to binding of specific molecules to the death receptors of the cell membrane, and the intrinsic pathway [120, 121].

A group of proteins, known as the Bcl-2 family, tightly regulate cell death and survival [14]. This family of proteins includes anti-apoptotic proteins, such as Bcl-2, Bcl-XL, Bcl-W, and pro-apoptotic proteins like Bax, Bad, Bid, Bim, Noxa, or PUMA [122]. The intrinsic pathways of apoptosis can be caspase-dependent or caspase-independent.

5.1 Intrinsic pathways of apoptosis

5.1.1 Caspase-dependent apoptosis

The ischemia-induced mitochondrial dysfunction and opening of the mitochondrial permeability transition pore (MPTP) lead to the release of cytochrome c and other pro-apoptotic factors such as apoptosis-inducing factor (AIF), high-temperature requirement protein A (HtrA2/OMI) [14], or second mitochondrion-derived activator of caspase/direct inhibitor of apoptosis-binding protein with low pI (SMAC/DIABLO [14]. Cytochrome c interacts with the cytosolic apoptotic-protease-activating factor-1 (Apaf-1) to form the apoptosome and, together with deoxyadenosine triphosphate, activates pro-caspase-9, which will cleave and activate caspase-3 [123]. Caspase-3 is a key mediator of apoptosis in animal models of stroke, its mRNA being upregulated 1 hour after the onset of focal ischemia [124]. It cleaves many proteins, among them PARP.

5.1.2 Caspase-independent apoptosis

Aside from upregulation of the pro-apoptotic Bcl-2 protein subfamily by ischemia [125], the mitochondrial dysfunction and opening of the MPTP lead to the release of AIF, which translocates to the nucleus fragments DNA and inhibits PARP, thereby accelerating cellular damage and destruction [126]. SMAC/DIABLO binds to X chromosome-linked inhibitor-of-apoptosis protein (XIAP) and triggers apoptosis by suppressing the anti-apoptotic activity of XIAP [127]. In addition, increased cytosolic calcium activates calpains and caspase-8, leading to cleavage and activation of Bcl-2 interacting domain (BID) [128], which translocates to mitochondria when the cell receives a death signal. Activated BID induces conformational changes in other pro-apoptotic proteins, such as Bax, Bad, Bcl-XS, and inactivate anti-apoptotic proteins like Bcl-2 or Bcl-XL [129].

The cells also have anti-apoptotic pathways, but these are overwhelmed after an ischemic insult. For example, inhibitor-of-apoptosis (IAP) proteins, including XIAP, NIAP, and others, bind and suppress the activity of caspases -3, -7, and -9 [130]. Various members of the Bcl-2 family (Bcl-2, Bcl-XL, Bcl-w) also try to suppress the apoptotic process [131]. CREB and NF-κB regulate a series of survival genes, such as Bcl-XL, and IAPs, the transcription factors in the PI3K/Akt signaling cascade, which also upregulates several neurotrophic factors, such as brain-derived neurotrophic factor (BDNF), insulin-like growth factor 1, or nerve growth factor (NGF) [15, 132]. Akt, whose activity is increased by superoxide dismutase 1 (SOD1, Cu-Zn SOD), also inhibits the induction of death genes such as Bim and phosphorylated Bad, making the PI3K/Akt signaling cascade a potential target for neuroprotective drugs [133].

5.2 The extrinsic pathway of apoptosis

This pathway also contributes to cell death after cerebral ischemia, being upregulated within 12 hours after the onset of focal cerebral ischemia and peaking 24 to 48 hours after the ischemic insult [121]. The binding of certain molecules initiates it on the surface receptors of the cells. These surface cell death receptors belong to the tumor necrosis factor receptor (TNFR) superfamily and include TNFR-1 and Fas. Forkhead 1, a transcription factor, stimulates the expression of several genes, such as Fas ligand (FasL), which binds to the Fas receptor and triggers recruitment of the cytoplasmic adaptor protein Fas-associated death domain protein (FADD) [121, 134]. FADD can bind to pro-caspase-8. The whole complex (FasL–Fas–FADD–procaspase-8) is also known as the death-inducing signaling complex (DISC). It is assembled within seconds of FasL binding to Fas, leading to activation of pro-caspase-8 and generation of caspase-8 [128]. Further, caspase-8 is released from the DISC complex and activates caspase-3 [121], leading to the execution phase of apoptosis. Fig. 3 (Ref. [135]) presents these pathways leading to apoptosis.

6. Neuroinflammation in cerebral ischemia/reperfusion injuries

The brain is an immune-privileged organ that is not readily accessible to immune cells due to the blood-brain barrier (BBB) [136]. The BBB has a layer of endothelial cells interconnected by tight junctions, placed on a basal membrane. Many pericytes are embedded [137] and are ensheathed on the abluminal aspect by astroglial endfeet [138].

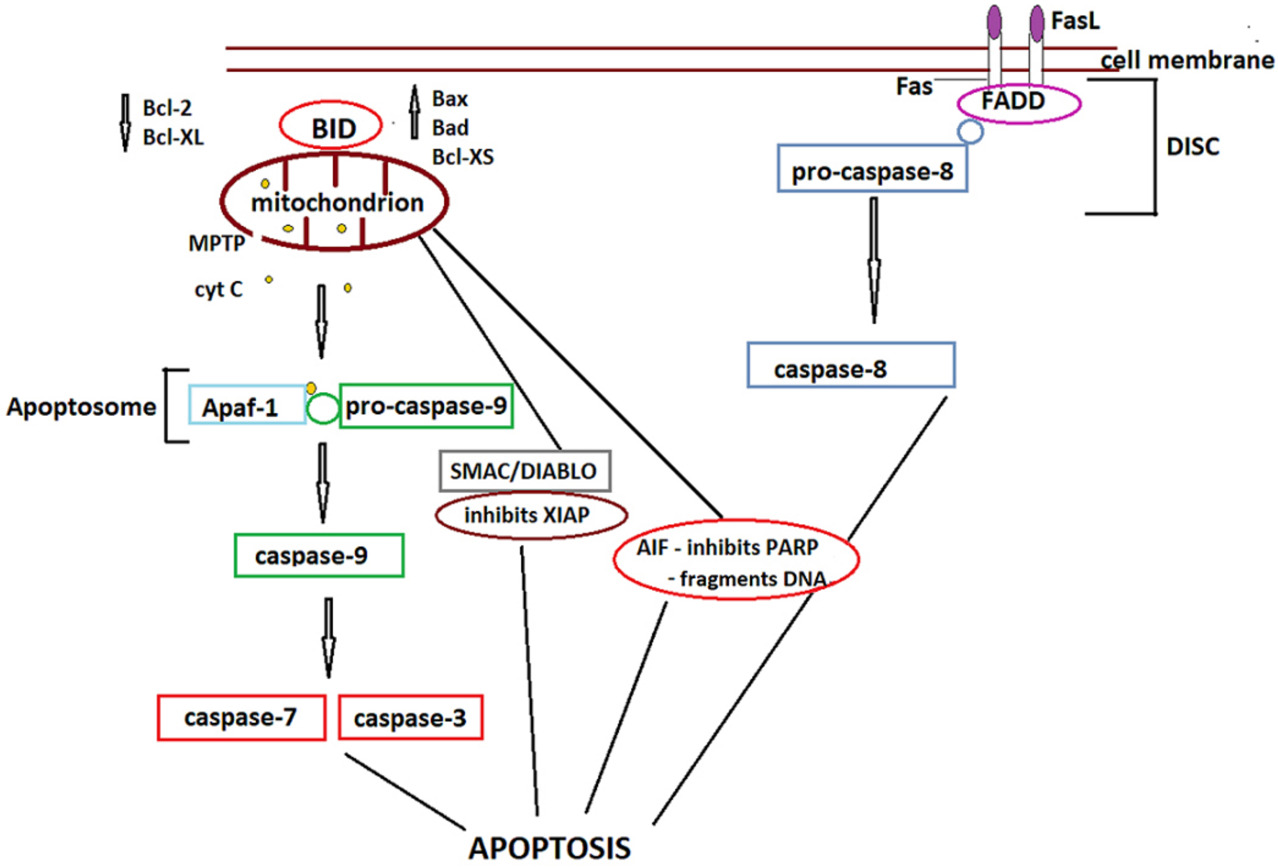

Fig. 3. Main pathways are leading to apoptosis. Opening of the mitochondrial permeability transition pore (MPTP) leads to the release of cytochrome C (cyt C), which together with the cytosolic apoptotic-protease-activating factor-1 (Apaf-1) activates procaspase-9. Active caspase-9 further activates caspases-3 and 7, leading to the execution phase of caspase-dependent apoptosis. The mitochondria also release apoptosis-inducing factor (AIF), high-temperature requirement protein A (HtrA2/OMI), as well as a second mitochondrion-derived activator of caspase/direct inhibitor of apoptosis-binding protein with low pI (SMAC/DIABLO), which inhibits the antiapoptotic X chromosome-linked inhibitor-of-apoptosis protein (XIAP), thereby leading to apoptosis. Various factors, such as increased cytosolic calcium-induced activation of calpains and caspase-8, cleave and activate Bcl-2 interacting domain (BID), which activates other pro-apoptotic proteins (Bax, Bad, Bcl-XS) and inactivates antiapoptotic proteins like Bcl-2 or Bcl-XL (caspase-independent apoptosis). The binding of Fas ligands (FasL) to Fas receptors recruits the cytoplasmic Fas-associated death domain (FADD) and pro-caspase-8, forming together the death-inducing signaling complex (DISC), which leads to activation of pro-caspase-8 and triggering of the extrinsic pathway of apoptosis. Adapted from [135].

6.1 Contribution of microglia to neuroinflammation after cerebral ischemic injury

Microglia is the primary immune cell of the central nervous system (CNS). The resting-state has a small cell soma and numerous processes that monitor the CNS's microenvironment [139]. Upon activation, microglia retract their processes and take on an amoeboid shape [140]. The main pathway for microglia activation is the NF-κB pathway, in which the inhibitory IκB protein, which is bound to NF-κB in the cytoplasm, is phosphorylated and degraded by IκB kinases allowing the nuclear translocation of NF-κB, where it promotes the transcription of many pro-inflammatory cytokine genes [141, 142]. However, a series of molecules released by damaged cells, such as ATP, heat shock proteins, S100 proteins, collectively known as damage-associated molecular patterns (DAMPs), contribute to this activation as well [4, 143].

Transient middle cerebral artery occlusion as short as 15 minutes in spontaneously hypertensive stroke-prone rats leads to microglial activation [144], after which these cells migrate toward the ischemic lesion and remain close to the neurons in a process called "capping", which helps quick removal of damaged neurons [145, 146]. The production of ROS via NADPH oxidase, of matrix metalloproteinases and cytokines, as well as activation of CD14 receptors by iNOS followed by the expression of toll-like receptor 4 (TLR4) in activated microglia increase its neurotoxic effects in the infarcted core as well as in the penumbra [147–151]. After transient middle cerebral artery occlusion in mice, microglial and macrophage infiltration peaks 48–72 hours after the ischemic insult [152]. Once arrived at the site of injury, microglia produce a series of pro-inflammatory cytokines, such as interleukin (IL)-1β and -6, tumor necrosis factor-α, as well as chemokines, like monocyte chemotactic protein-1 and macrophage inhibitory factor-1α, which recruit leuko-

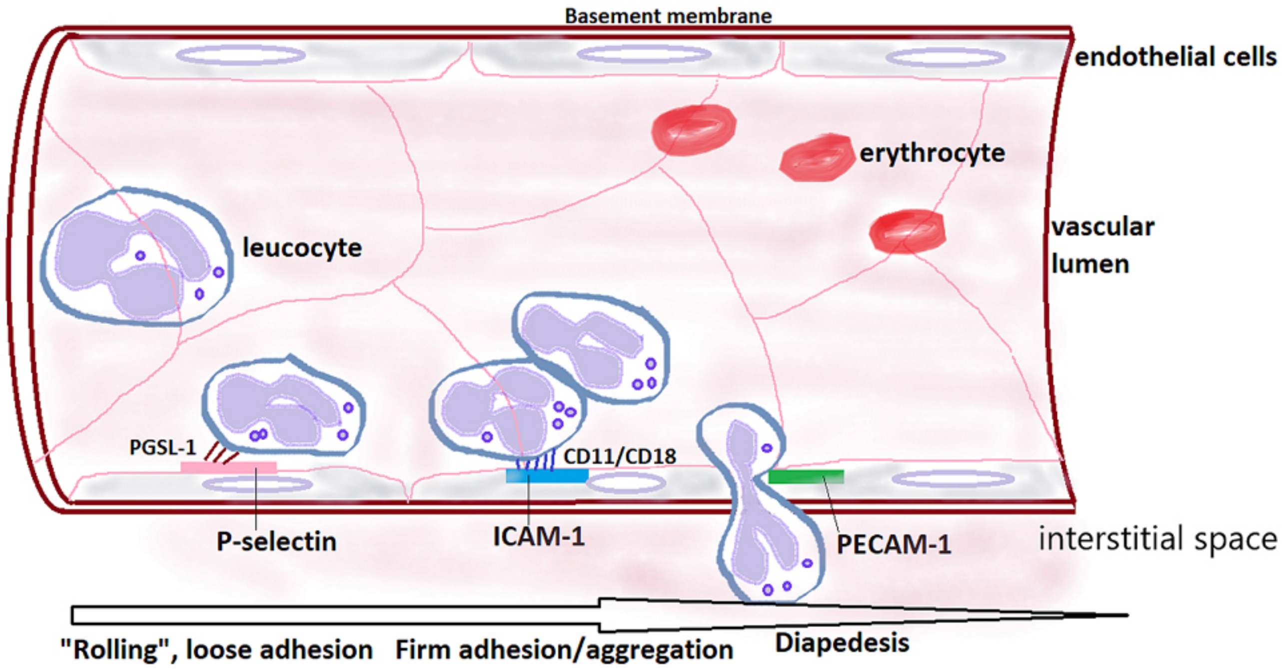

Fig. 4. Leucocyte diapedesis. Leucocytes interact with endothelial cells expressing P selectins through P-selectin glycoprotein 1 (PSGL-1), leading to their "rolling" on the endothelial surface. Interaction of leucocyte integrins CD11a/CD18 and CD11b/CD18 with intercellular adhesion molecule 1 (ICAM-1) leads to firm adherence and aggregation of leucocytes. Diapedesis of leucocytes is facilitated by platelet-endothelial cell adhesion molecule 1 (PECAM-1). Adapted from [163].

cytes to the injured parenchyma [89], which, in turn, will release proteases and oxygen radicals which will potentiate tissue destruction [153]. Inhibiting microglial activation (with 2% isoflurane) in rats subjected to transient focal cerebral ischemia resulted in reduced infarct size and attenuated apoptosis [154].

However, microglia play a dual role after ischemic stroke, secreting pro-inflammatory as well as anti-inflammatory factors [147]. Research has shown that impaired microglial activation increased infarct size and potentiated neuronal apoptosis following ischemia [155]. Depletion of microglia with PLX3397, a dual colony-stimulating factor-1 inhibitor, increased infarct size and worsened the neurological deficits [156]. In addition, microglia produce a variety of neurotrophic factors which promote neuroplasticity and neurogenesis [157]. Thus, it appears that different subsets of microglial cells have different roles following cerebral ischemia [145].

6.2 Leukocytes in cerebral I/R injury

Leukocytes are among the first blood-derived immune cells entering the brain after cerebral ischemia, peaking at 48–72 hours and rapidly declining afterward [143]. After minutes to hours after the ischemic insult, ROS, cytokines and chemokines released by the damaged tissue induce the expression of adhesion molecules on leukocytes and cerebral endothelial cells [145, 158]. Cytokines such as tumor necrosis factor (TNF)-α or interleukin (IL)-1β lead to translocation of P-selectin to and the expression of intercellular adhesion molecule (ICAM)-1 and vascular cell adhesion molecule (VCAM)-1 on the endothelial cell surface [136]. This facilitates the rolling of leukocytes on the vascular wall, a process following which leukocytes change shape and become flattened, parallel with a directional polarization and redistribution of adhesion, signaling and receptor proteins toward an edge from which processes extend [4]. After the interaction of endothelial P-selectin with the receptor P-selectin glycoprotein ligand-1 (PSGL-1), the leukocyte β2 integrins CD11a/CD18 and CD11b/CD18 interact with ICAM-1, leading to firm adhesion of leukocytes to the endothelial cells [158, 159]. Further, expression of platelet-endothelial cell adhesion molecule-1 (PECAM-1) along the endothelial cell junction as well as expression of the junctional proteins JAM-A (junctional adhesion molecule-A) and JAM-B by pericytes, facilitate neutrophil diapedesis across the BBB [160, 161]. Once in the tissue, leukocytes produce a series of factors that exacerbate tissue injury, such as ROS, IL-1, IL-6, IL-12, TNFα, and proteases [4, 162]. Fig. 4 (Ref. [163]) presents the stages of leucocyte-endothelium interaction leading to leucocyte infiltration of the tissue damaged by ischemia.

6.3 Lymphocytes in cerebral I/R injury

Lymphocytes have a less important contribution in cerebral ischemic injury; the mechanisms are mainly related to the innate T-cell functions [164]. IL-17 secreting T cells aggravate ischemic injury [165], as do natural killer T cells, while IL-10-secreting regulatory lymphocytes (Tregs) have neuroprotective activity by downregulating postischemic inflammation [166, 167].

6.4 Inflammatory mediators

6.4.1 Cytokines

Cytokines are small polypeptides (8–26 kDa), normally expressed at very low levels, regulating immune responses [168].

IL-1β exacerbates cerebral injury after an ischemic insult [116] and significantly contributes to the disruption of BBB. Systemically administered lipopolysaccharides induce the production of pro-IL-1β, which is cleaved by caspase-1 to form IL-1β, which will subsequently disrupt the BBB [169, 170]. IL-1β administered to rats increased the magnitude of brain injury [171], while IL-1β-deficient mice had smaller volume infarcts than wild-type mice [172]. Treatment with or overexpression of IL-1 receptor antagonists also reduced infarct size [173, 174].

Tumor necrosis factor (TNF)-α is upregulated after cerebral ischemia, and protein levels are increased by 3 hours after ischemia peaking up to 5 days after the insult [175]. The cytokine induces the release of matrix metalloproteinase-9 (MMP-9) from pericytes leading to increased permeability of the BBB [176]. However, TNF-α has also been shown to be neuroprotective and involved in ischemic preconditioning [177]. It appears that this dual role depends on the source of TNF-α, microglial-derived TNF-α being neuroprotective in stroke [168].

IL-10 is an anti-inflammatory cytokine upregulated in ischemic stroke, peaking 3 days after the onset [178]. Animal research with intraventricular administration of IL-10 or adenoviral delivery of the IL-10 gene confirmed the neuroprotective effect of this cytokine [179, 180].

Another neuroprotective cytokine is interferon-β (IFN-β), long used as an immunomodulatory treatment in multiple sclerosis, which reduces the MMP-9 levels and diminishes the BBB disruption [181, 182]. Experimentally, IFN-β downregulated ICAM-1 expression on cerebral endothelial cells and attenuated BBB disruption and neutrophil infiltration in rats [183].

6.4.2 Chemokines

Chemokines are low molecular weight proteins (8–10 kDa) involved in cellular activation and leukocyte recruitment.

Monocyte chemoattractant protein-1 (MCP-1) directly increases the permeability of the BBB by causing tight-junction proteins to redistribute in endothelial cells [184] and recruits monocytes and activated lymphocytes into the brain after an ischemic insult [185].

Other chemokines upregulated in the first 3 hours after stroke are microglial response factor-1 (MRF-1), fractalkine, and macrophage inflammatory protein 1 (MIP-1), which all contribute to infiltration of the injured tissue with inflammatory cells and weaken the BBB [145].

However, stromal cell-derived factor 1 (SDF1), also known as C-X-C motif chemokine 12 (CXCL12), maybe neuroprotective, being found increased in the ischemic penumbra and facilitating homing of bone marrow stromal cells to the tissue injured by ischemia [186], thereby reducing the size of infarction and enhancing neural plasticity [187].

6.4.3 Matrix metalloproteinases

Although this family of enzymes is not a part of neuroinflammation, due to their significant involvement in BBB disruption, their course in acute ischemic stroke will be briefly discussed. MMPs are constitutive enzymes, such as MMP-2 and MMP-14, or inducible ones, like MMP-3 and MMP-9 [145]. The expression of MMP-9 increases significantly within 24 hours from the onset of ischemia in rats [188] and, together with tissue plasminogen activator, disrupts the BBB leading to hemorrhagic transformation [189]. Experimentally, MMP inhibition alleviates hemorrhage and brain edema and can also reduce infarct size [190]. On the other hand, plasma levels of MMP-3 were found to increase in patients with better functional and motor recovery [191], highlighting the dual role of these enzymes in stroke pathogenesis and recovery.

7. Concluding remarks

Despite the large amount of research focusing on the molecular pathophysiological mechanisms of ischemia/reperfusion injuries, the translation of these findings into clinically applicable therapies has been disappointing. As revascularization therapies continue to improve, gain popularity, and increase their therapeutic time window, reperfusion injuries are expected to increase in frequency. Clinical therapeutic advances have been hampered by coexisting risk factors that can prevent activation of cell survival programs and the dual nature of many of the described pathophysiological cascades, making correct timing an issue in their application. It is more likely that combined approaches, with concomitant employment of revascularization treatment, antioxidant, neuroprotective, and vasoprotective agents, will yield satisfactory results and extend the time window for efficient ischemic stroke treatment for the benefit of an expanding proportion of the aging population at risk for stroke.

Abbreviations

ADP, adenosine diphosphate; AIF, apoptosis-inducing factor; Akt, protein kinase B; AMPARs, α-amino-3-hydroxy-5-methylisoxazole-propionic acid receptors; APAF-1, apoptotic protease activating factor-1; ASK1, apoptosis signal-regulating kinase 1; ATP, adenosine triphosphate; Bad, Bcl-2-associated agonist of cell death; Bax, Bcl-2 associated X protein; BBB, blood brain barrier; Bcl-2, B cell lymphoma 2; BDNF, brain-derived neurotrophic factor; Bid, BH3-interacting domain death agonist;

Bim, Bcl-2-interacting mediator of cell death; CAMKs, Ca^{2+}/calmodulin-dependent protein kinases; CAT, catalase; CNS, central nervous system; COX, cyclooxygenase; CREB, cAMP-response element binding protein; CYPs, cytochrome P450 enzymes; DAMPs, damage-associated molecular patterns; DISC, death-inducing signaling complex; DNA, deoxyribonucleic acid; Drp1, dynamin-related protein 1; EAATs, excitatory amino acid transporters; ECASS, European Cooperative Acute Stroke Study; END, early neurological deterioration; ERK, extracellular signal-regulated kinase; FADD, Fas-associated death domain; Fas, a type I transmembrane protein containing a death domain in its cytoplasmic region; FasL, Fas ligand; GPX, glutathione peroxidase; GSK3β, glycogen synthase kinase 3β; GTP, guanosine triphosphate; HETE, hydroxyeicosatetraenoic acid; HtrA2/OMI, high-temperature requirement protein A2 (also known as OMI); IAP, inhibitor-of-apoptosis protein; ICAM, intercellular adhesion molecule; IL, interleukin; I/R injuries, ischemia/reperfusion injuries; JAM, junctional adhesion molecule; JNK, c-Jun N-terminal kinase; LOX, lipoxygenase; MAPK, mitogen-activated protein kinase; MCP-1, monocyte chemoattractant protein-1; mRNA, messenger RNA; MIP-1, macrophage inflammatory protein 1; MMP, matrix metalloproteinase; MPTP, mitochondrial permeability transition pore; MRF-1, microglial response factor-1; MRI, magnetic resonance imaging; NAD, nicotinamide adenine dinucleotide; NADH, reduced NAD; NADP, nicotinamide adenine dinucleotide phosphate; NADPH, reduced nicotinamide adenine dinucleotide phosphate; NF-κB, nuclear factor κB; NGF, nerve growth factor; NHE, Na^+/H^+ exchanger; NIAP, neuronal apoptosis inhibitor protein; NIHSS, National Institute of Health Stroke Scale; NMDARs, N-methyl-D-aspartate receptors; NO, nitric oxide; NOS, nitric oxide synthase, with 3 isoforms; eNOS, endothelial NOS; iNOS, inducible NOS; nNOS, neuronal NOS; NOX, NADPH oxidase; p38, p53, proteins 38, 53; PARP, poly (ADP-ribose) polymerase; PECAM-1, platelet-endothelial cell adhesion molecule-1; PI3K, phosphoinositide-3-kinase; PPARs, peroxisome-proliferator-activated receptors; PSGL-1, P-selectin glycoprotein ligand-1; PUMA, p53 upregulated modulator of apoptosis; QH_2, ubiquinol; Ras, a small GTPase; RNA, ribonucleic acid; ROS, reactive oxygen species; SDF-1, stromal cell-derived factor 1; SMAC/DIABLO, second mitochondrion-derived activator of caspase/direct inhibitor of apoptosis-binding protein with low pI; SOD, superoxide dismutase; TLR4, toll-like receptor 4; TNF, tumor necrosis factor; TNFR, tumor necrosis factor receptor; Tregs, regulatory lymphocytes; VCAM, vascular cell adhesion molecule; XIAP, X chromosome-linked inhibitor-of-apoptosis protein; XO, xanthine oxidase.

Author contributions

AJ conceptualized the study; AJ and IAA analyzed the literature and synthesized it; AJ wrote the first draft of the paper. Both authors agreed on the submitted material.

Ethics approval and consent to participate

Not applicable.

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Conflict of interest

The authors declare no conflict of interest.

Appendix

Epidemiology of early neurologic deterioration (END)

Worsening of the neurological status early in ischemic stroke is a common finding with serious short- and long-term consequences for the patient. Initially termed "stroke in progression", or "stroke in evolution", the accurate definition of the term in various trials depends on the neurological scale used to quantify the neurological deficit:

• The ECASS (European Cooperative Acute Stroke Study) I trial used the Scandinavian Neurological Stroke scale and defined early progression as a decrease of $\geq$2 points in the consciousness or motor power scores or a decrease of $\geq$3 points in the speech scores in the first 24 hours after admission [192].

• In the Oxfordshire Community Stroke Project, early deterioration was defined as a decrease of $\geq$1 point in the Canadian Neurological Scale in patients with partial or total anterior circulatory infarcts and lacunar strokes and as $\geq$1 point decrease on the Rankin score in patients with posterior circulatory infarcts in the first 7 days from stroke onset [193].

• More recently, an increase of $\geq$2 points on the National Institute of Health Stroke Scale (NIHSS) score or stroke-related death in the first 5 days after admission were considered to indicate early neurological deterioration (END) [194].

The incidence of END varies in different trials, ranging between 13% and one-third of patients [195–198] but has been reported by some researchers to be as high as 43% [199].

Predisposing factors for END are:

• Hyperglycemia [198–200], which leads to increased concentrations of lactate and acidosis in the penumbral area [201], worsens mitochondrial function in the penumbra [202] and predicts poor outcome [203].

• Low systolic and diastolic blood pressure levels (<100/70 mm Hg) [204], which in the setting of acute ischemic stroke, low blood pressure can be caused by heart disease with impaired left ventricular function and low cardiac output, dehydration, infections with septic states, or aggressive antihypertensive medication [205, 206]. Due to acidosis and hypoxia of tissues in the penumbral area, cerebral

Table 1. Conditions and mechanisms by which these conditions lead to END.

Conditions leading to END	Frequency	Mechanisms leading to END
Hemorrhagic transformation	10%	Oxidative stress, neuroinflammation, matrix metalloproteinases – leading to weakening of the blood brain barrier and destruction of the vascular tissue, allowing blood to spill into the infarcted tissue
Cerebral edema	19%	Anaerobic metabolism leading to tissue acidosis, failure of ionic pumps, leading to cytotoxic edema; compression of the vasculature with further impairment of glucose and oxygen supply, propagating in a cascade, leading to increase in intracranial pressure, shifting of brain tissue, herniation syndromes
Failure of collaterals	Most frequent cause	Extended area of tissue with oxygen and glucose deprivation, increased oxidative stress, ischemic and neuroinflammatory cascades leading to tissue infarction
Arterial reocclusion	34%	Reignition of the ischemic cascade, increased oxidative stress, neuroinflammation
Recurrent stroke	11%	New areas of infarction, cerebral edema, oxidative stress
Prolonged seizures	5%	Excitotoxicity, oxidative stress
General conditions, infections		Variable, depending on the cause

autoregulation is compromised, and perfusion relies on systemic blood pressure. Thus, hypotension decreases collateral blood flow and contributes to the extension of the infarcted area [207]. In fact, in the International Stroke Trial, the death rate increased by 17.9% for every 10 mm Hg of systolic blood pressure below 150 mm Hg [208].

• Elevated levels of high-sensitivity C reactive protein [209], or serum cystatin C [210].

• The presence of large vessel occlusion [211].

• A large perfusion-weighted imaging (PWI)/diffusion-weighted imaging (DWI) mismatch (commonly used in trials to identify tissue at risk after acute cerebral ischemia) [212, 213].

Mechanisms contributing to END may include the following, summarized in Table 1.

• Clot propagation with obstruction of more collaterals, although never demonstrated, was considered in the past to be the main cause of the stepwise worsening of the neurological deficit in acute ischemic stroke patients, leading to a long debate as to the use of anticoagulants for the treatment of stroke in progression [214–216]. More recently, MRI studies have shown that patients are more likely to have large vessel occlusions with failure of collateral circulation [217, 218].

• Hemorrhagic transformation is involved in about 10% of END [195]. It has a wide range of severity, from small suffusions and petechiae to large hematomas occurring in the infarcted area with a mass effect on the surrounding tissue [219]. Factors leading to hemorrhagic transformation include oxidative stress and reperfusion injuries, which, in turn, lead to neuroinflammation, leukocyte infiltration, and extracellular proteolysis, culminating with the destruction of the basal lamina and the tight endothelial junctions [219, 220]. It can have various radiological patterns, and several grading systems have been proposed. The ECASS grading system differentiates among hemorrhagic infarction (HI), further subdivided into small petechiae along the margins of the infarcted area (HI1) or confluent petechiae in the infarcted area, without any mass effect (HI2), and parenchymal hematomas (PH), with blood clots in $\leq$30% of the infarcted area with some mass effect (PH1), or PH2, describing blood clots in >30% of the infarcted area with significant space-occupying effect [221]. Although it is a rather frequent complication after recanalization therapies, with incidences reaching as high as 6% after intravenous thrombolysis or 8% after mechanical thrombectomy [222, 223], only large parenchymal hematomas with mass effect are associated with neurological worsening or even death [224].

• Re-occlusion of a recanalized artery occurs in about 34% of thrombolysed patients and accounts for two-thirds of deteriorations following initial improvement [225].

• Cerebral edema is involved in about 19% of END, especially following occlusion of large arteries [195].

• Recurrent stroke, occurring either in the original arterial territory or in a remote location, causes about 11% of ENDs [195].

• Seizures are common following large cortical infarctions. Although they usually cause only a temporary worsening, if prolonged, they can lead to END in about 5% of patients [226].

However, as already mentioned, most clinicians ascribe END to ischemia/reperfusion injuries, which can lead to additional cerebral injury, weaken the blood-brain barrier (BBB) and cause hemorrhagic transformation, potentiate cerebral edema, and contribute to the "no-reflow phenomenon" in which despite clot removal, efficient microvascular perfusion is not achieved. Angiographic studies in ischemic stroke patients confirmed the lack of reperfusion, although the patency of large vessels was restored [227].

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