Review

Oxidative Stress in Cerebral Ischemia/Reperfusion Injury

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Abstract

Oxidative stress in cerebral ischemia/reperfusion injury (CIRI) involves reactive oxygen and nitrogen species (ROS and RNS). Despite efficient antioxidant pathways in the brain, hypoxia triggers the production of oxygen free radicals and downregulates ATP, which leads to

oxidative stress. Sources of free radicals during CIRI include Ca^{2+}-dependent enzymes, phospholipid degradation and mitochondrial enlargement. Upon reperfusion, the abrupt increase of oxygen triggers a massive radical production via enzymes like xantin oxidase (XO), phospholipase A2 (PLA2) and oxide synthases (OS). These enzymes play an essential role in neuronal damage by excitotoxicity, lipoperoxidation, nitrosylation, inflammation and programmed cell death (PCD). Endothelial nitric oxide synthase (eNOS) decreases as compared to neuronal nitric oxide synthase (nNOS). This is associated with neuronal damage, endothelial inflammation, apoptosis and oxidative stress. Strategies promoting activation of eNOS while inhibiting nNOS could offer neuroprotective benefits in CIRI. Understanding and targeting these pathways could mitigate brain damage in ischemia/reperfusion events. Clinically, tissue plasminogen activator (t-PA) has been shown to restore cerebral blood flow. However, serious side effects have been described, including hemorrhagic transformation. Different treatments are currently under investigation to avoid I/R injury. Baicalin has been reported as a potential agent that could improve t-PA adverse effects, which have to do with peroxynitrite synthesis and matrix metalloproteinase (MMP) expression. In this review, CIRI and interventions in oxidative stress are addressed. Special attention is paid to efficient antioxidant mechanisms in the brain and the production of free radicals, especially nNOS-derived nitric oxide (NO). The primary purpose is to describe accessible radical pathways with the activity of Ca^{2+}-dependent oxidative enzymes, leading to membrane phospholipids and mitochondrial breakdown.

Acute stroke, often referred to as cerebrovascular accident, is one of the leading causes of mortality and disability worldwide. Stroke has been categorized into two main types: Ischemic and Hemorrhagic. Ischemic type is responsible for 85% of the cases [1, 2]. Restoration of blood flow to previously ischemic tissues leads to the paradoxical phenomenon known as Ischemia/Reperfusion Injury (I/RI). Pathophysiology and damaging mechanisms in cerebral Ischemia/Reperfusion injury (CIRI) have been described in the literature. These include but are not limited to energetic metabolism impairment, cellular acidosis, synthesis of replicating excitotoxic amino acids, cellular calcium homeostasis, free radical production, and apoptotic gene activation [3].

Although there are efficient antioxidant mechanisms in the brain, these mechanisms are overcome under excessive production of free radicals, and oxidative stress is established [4, 5]. Both oxygen free radicals (OFR) and reactive nitrogen species (RNS) are vital mediators of ischemia/reperfusion (I/R) injury. Their primary damaging mechanism is oxidative injury to various biological molecules, interfering with their function [6]. Irreversible injury is produced through mitochondrial dysfunction and failure of energy metabolism [3]. Oxygen free radicals involve superoxide anion [O_2^-], peroxide [O_2^{-2}], hydroxyl radical [$\cdot OH$] and hydrogen peroxide [H_2O_2]. They all are collectively known as reactive oxygen species (ROS). ROS and RNS are reactive oxygen and nitrogen species (RONS) [7].

During CIRI, calcium-dependent oxidative enzymes, membrane, and mitochondria phospholipid degradation are the primary sources of free radicals [8]. While injury resulting from Ischemia/Reperfusion can alter protective mechanisms such as glutathione, coenzyme Q10, superoxide dismutase, and glutathione peroxidase. Thus exacerbating the production of ROS [9]. Free radical production begins early in ischemia and increases during reperfusion [8]. Upon reperfusion, the sudden rise in oxygen levels is a substrate for several previously activated oxidative enzymes. Some of the most important are Xanthine Oxidase (XO), phospholipase A2 (PLPA$_2$) and neuronal nitric oxide synthase (nNOS). Xanthine Oxidase plays a key role in ROS production, such as Superoxide Anion (O$_2^-$) and Hydrogen Peroxide (H$_2$O$_2$). Phospholipase A2 releases arachidonic acid during membrane phospholipid degradation, which can further increase the production of ROS and lipid second messengers (eicosanoids) [9]. The latter are active promoters of inflammation through lipoxygenase and cyclooxygenase pathways, causing endothelial injury and increasing blood-brain barrier (BBB) permeability through lipid and oxidized protein reactivity [3, 10]. Neuronal nitric oxide synthase produces nitric oxide (NO), which, depending on the oxidative environment, can lead to NO·, nitrogen dioxide (NO^2) and peroxynitrite (ONOO$^-$) [11]. Within the mitochondria, hypoxia increases O$_2$ and ONOO$^-$ synthesis, mostly in respiratory complex III and IV [12, 13]. During ischemic conditions, ATP levels are insufficient to maintain cellular functions. This triggers H/Na$^+$ exchangers, which pump hydrogen into the extracellular space, further increasing ROS (Figure 1) [9].
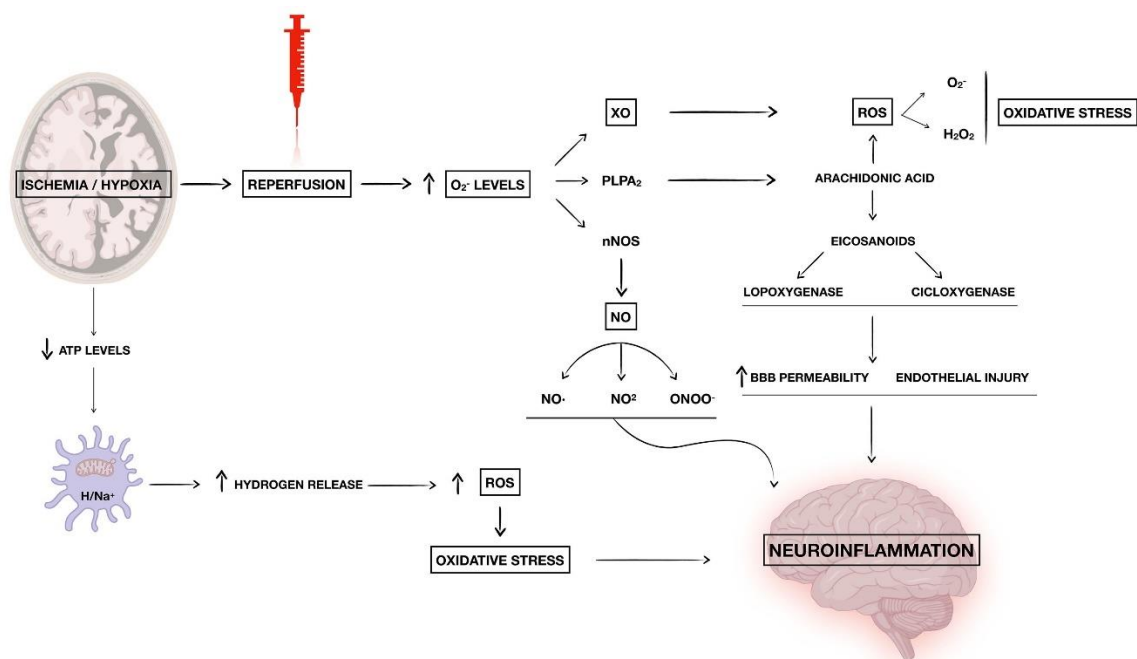

Figure 1 Roles of Xanthine Oxidase, phospholipase A2, and neuronal nitric oxide synthase in Cerebral Ischemia/Hypoxia leading to Neuroinflammation. ROS, Reactive Oxygen Species; NO, Nitric Oxide; nNOS, neuronal Nitric Oxide Synthase; O$_2$, Oxygen; ATP, Adenosine Triphosphate; Na, Sodium; H, Hydrogen; XO, Xanthine Oxidase; PLPA$_2$, Phospholipase A2; O$_2^-$, Superoxid Ion; H$_2$O$_2$, Hydrogen Peroxide; KWFHWR; NO$_2$, nitrogen dioxide; ONOO$^-$, peroxynitrite; BBB, blood-brain barrier.

Nitric oxide (NO) is a gaseous molecule that can be present either as a nitrogen-free radical or as a nitrous ion, depending on the cell redox status. This molecule can be produced by three different

nitric oxide synthases (NOS). And three isoforms have been described and cloned: neural or brain NOS (nNOS/bNOS; type 1), endothelial NOS (eNOS, type 3), and inducible NOS (iNOS, type 2) [7]. All three NOS isoenzymes use nicotinamide adenine dinucleotide phosphate (NADPH) and molecular oxygen as substrates. Neuronal and endothelial NOS's are constitutively expressed and their activity is Ca^{2+}/calmodulin dependent [9, 10]. A series of complex pathophysiological events result from CIRI. These include but are not limited to a burst in ROS, Ca^{2+} overload and a disrupted mitochondrial architecture. Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) is activated by autophosphorylation and regulates various physiological functions. Synthesis of neurotransmitters and current membranes are just some of them. Calcium excess results from a decline in ATP synthesis during cerebral ischemia and hypoxia. Oxygen influx, upon reperfusion, restores aerobic ATP generation, fuels ROS production, and removes hydrogen ions from the extracellular space. Excitotoxicity and cerebral edema are promoted by Ca^{2+} influx. Furthermore, Ca^{2+} excess is absorbed by mitochondria. This phenomenon develops the mitochondrial permeability transition pore (mPTP), which triggers cell death pathways (Figure 2) [13-15]. Nitric oxide plays a key role in different physiological processes. Vascular tone regulation, immunomodulation and neurotransmission are just some of these processes [14, 15]. Nitric oxide is less reactive than most of the ROS. However, the NO and superoxide anion (O_2^-) reaction leads to $ONOO^-$ a potent lipoperoxidant and protein nitrosylating agent [16].
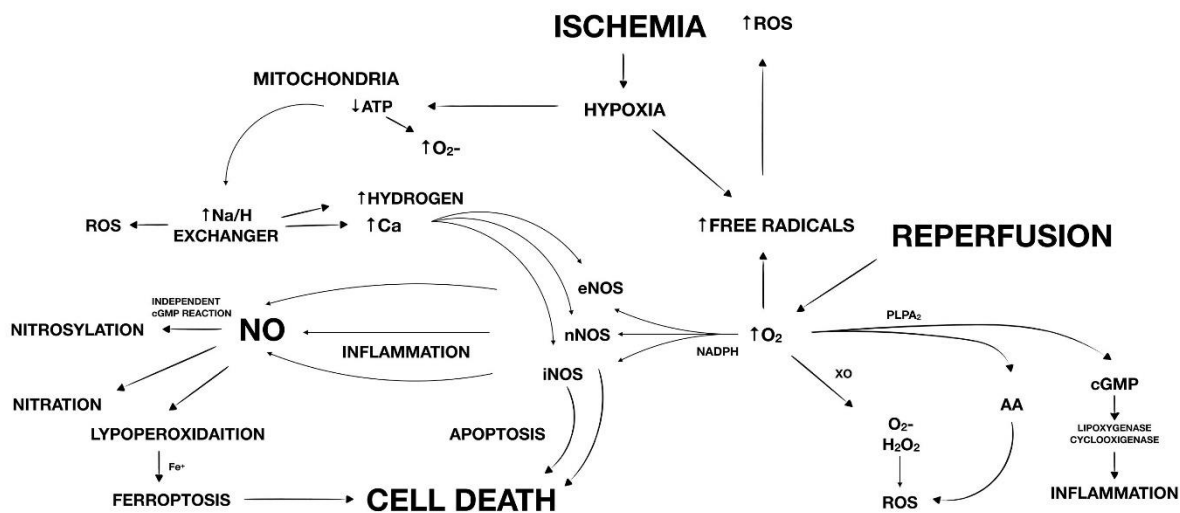

Figure 2 Oxidative stress in cerebral ischaemia/reperfusion. On reperfusion, abrupt increase of oxygen triggers a massive radical production. The nNOS-derived NO overproduction is associated with excitotoxicity, neural apoptosis, and oxidative stress. The reaction between NO and superoxide anion (O_2^-) leads to peroxynitrite ($ONOO^-$), a potent lipoperoxidant and protein nitrosylating agent. ROS, Reactive Oxygen Species; NO, Nitric Oxide; eNO, endothelial Nitric Oxide Synthase; nNOS, neuronal Nitric Oxide Synthase; iNO inducible Nitric Oxide Synthase; O_2, Oxygen; ATP, Adenosine Triphosphate; Ca^+, Calcium; Na, Sodium; H, Hydrogen; Fe^+, iron; NADPH, Nicotinamide Adenine Dinucleotide Phosphate; XO, Xanthine Oxidase; PLPA$_2$, Phospholipase A2; AA, Araquidonic Acid; O_2^-, Superoxide Ion; H_2O_2, Hydrogen Peroxide; GMPc, Cyclic Guanosine Monophosphate.

Nitric oxide is well known for its capacity to stimulate cGMP production when interacting with the ferrous ion of the soluble guanylate cyclase hem group. However, some of the effects do not rely on cGMP activity. This is the case of lipid peroxidation, oxidative and protein nitration [14]. Nitrosylation involves covalently adding a nitrogen monoxide (NO) group into an organic molecule without affecting its charge. Therefore, different nitroso groups are categorized as C-nitroso, N-nitroso, O-nitroso, or S-nitroso compounds. The most common reaction is S-nitrosylation between cysteine thiol groups facilitated by the interaction of sulfur and NO. Nitration involves introducing a nitro group (NO_2) into proteins, particularly tyrosine residues, which results in 3-nitrotyrosine. This specific protein nitration uncovers the disturbance of NO signaling towards nitro-oxidative stress [17]. Free radical peroxidation of polyunsaturated fatty acids (lipid peroxidation), such as linoleic acid and arachidonic acid, is associated with a form of programmed cell death (PCD) known as "ferroptosis". This PCD occurs within the first hour of Ischemia due to high iron levels. And it is considered one of the various causes of cell injury/death [18].

The role of NO in CIRI has been widely studied since it was first described in the 1990s [15]. However, contradictory evidence has been reported regarding its protective or detrimental effects on CIRI. Evidence suggests that NO effect may vary depending on concentration, region, source, and medium [19]. Under normal conditions, low NO concentrations are maintained through the eNOS pathway. The primary purpose is to regulate blood flow and protect the vascular endothelium and brain parenchyma from inflammatory, procoagulant, and oxidative stimuli [14, 15]. However, during I/RI, eNOS activity decreases compared to nNOS [20]. Animal models of CIRI have shown that eNOS downregulation is associated with increased brain damage, endothelial inflammatory phenotype, oxidative stress, and reduced FSC [21-23]. Meanwhile, nNOS has a significant role in cell damage. This isoenzyme's (nNOS) activity upregulates once Ischemia is established and during the first hour post-reperfusion [20, 22]. The nNOS-derived NO overproduction is associated with excitotoxicity, neural apoptosis, and oxidative stress [24]. Right after reperfusion, hydrogen excess in extracellular space moves back into the cell, carrying calcium (Ca^+) ions. While the mitochondria regulate calcium levels, massive influx results in organelle swelling, which leads to transient permeability. This phenomenon triggers PCD, which leaves unprotected neurons susceptible to injury [25, 26]. However, nNOS downregulation has been associated with neuron protection [20, 27-31]. Inducible nitric oxide synthases (iNOS) are expressed around 12 hours after reperfusion. Overproduction of NO by this enzyme has also been identified as harmful in conditions of cerebral I/R [20, 31-33]. Therefore, promoting eNOS activity while blocking nNOS and iNOS could bring neuroprotective effects under conditions of CIRI (Figure 1, Figure 2).

Inflammatory cytokines have also been reported to have a role in Ischemia/reperfusion injury (I/RI) [15]. However, their effect can be either neuroprotective or neurotoxic. During CIRI, IL-6 is upregulated, which triggers an inflammatory response [3]. Nevertheless, excessive production of this cytokine has been demonstrated to alter the BBB, which results in secondary edema and injury to brain tissues [34]. A correlation between the production of free radicals and damage to the central nervous system has been demonstrated. Therefore, investigations have focused on inhibiting/blocking ROS pathways to mitigate oxidative stress in the brain [3]. In Asian Countries, Edaravone® is the most common drug utilized in conditions of CIRI. This drug has anti-apoptotic, anti-necrotic, and anti-inflammatory properties [3]. Therefore, it improves mitochondrial edema and eNOS upregulation and reduces free radical synthesis, stroke size, and delayed neuronal damage [3]. The FDA-approved tissue plasminogen activator (tPA) has been described as a clinical treatment for

ischemic stroke. It spontaneously restores cerebral blood flow when administered within the first 4.5 hours [35]. However, delayed tPA treatment can have serious side effects [35, 36]. An increased risk of hemorrhagic transformation has been reported when indicated after 4.5 hours of ischemic stroke [35]. That is, delayed tPD treatment induces peroxynitrite synthesis, which promotes Matrix metalloproteinase (MMPs) activation. MMPs mediate both BBB disruption and hemorrhagic transformation [35, 36]. Metalloproteinases are a group of zinc-dependent proteolytic enzymes mainly expressed in the brain. Activation of MMPs results in the breakdown of tight junctions (TJs) and extracellular matrix (ECM) adjacent to cerebral blood vessels and neurons. This biomolecular degradation finally leads to endothelial hyperpermeability and BBB disruption. The peroxynitrate effect was confirmed following the use of Baicaline. Hemorrhagic transformation was increased by inhibiting MMPs on an ischemic stroke model after delayed tPA treatment [35].

Specific drugs capable of reducing neuroinflammation have been unsuccessful since irreversible mitochondrial damage is the result of a wide range of pathways [13]. That is, Ca2+ overload, mitochondrial DNA deficiencies and oxidative stress characterize the pathophysiology of CIRI. Antioxidants provide neuroprotective effects by eliminating reactive oxygen species while inhibiting lipid peroxidation. Accordingly, future research should be aimed at identifying innovative drugs capable of eliminating free radicals and with good synergy with anti-inflammatory medications. Natural products known for their antioxidant, anti-inflammatory, and free radical scavenging properties in treating cerebrovascular diseases have been investigated in animal models of ischemia/reperfusion injury [37]. Ginkgo Biloba significantly reduced nitric oxide levels in an experimental muscle ischemia/reperfusion injury model. Since radical scavenging drugs are expensive, investigating natural anti-inflammatory drugs becomes an exciting line of research [38]. Agents targeting mitochondrial dysfunction and programmed cell death would also be promising neuroprotective therapeutic strategies. Another critical element is to continue studying the pathophysiological mechanisms involved in acute stroke. Targeting neuroinflammation involved in the ischemic cascade has become an attractive therapeutic intervention [39]. However, protective strategies are time-dependent when inhibiting acute stroke pathways. Interventions occurring at the wrong time could worsen acute stroke pathophysiology. This is the case of the stroke-heart syndrome [40]. Treatment interventions rely primarily on thrombolytic intravenous drugs. Nevertheless, the time frame between acute ischemic stroke and acute myocardial infarction is different. Therefore, studying brain-heart interactions and signal pathways has become another important line of investigation.

Author Contributions

All authors contributed equally to the conception, literature review, drafting, the overall content and revising of the article. We all approved the version of the manuscript to be published.

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Competing Interests

The authors have declared that no competing interests exist.

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