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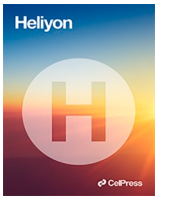

Review article

Crosstalk between autophagy and ferroptosis mediate injury in ischemic stroke by generating reactive oxygen species

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ABSTRACT

Stroke represents a significant threat to global human health, characterized by high rates of morbidity, disability, and mortality. Predominantly, strokes are ischemic in nature. Ischemic stroke (IS) is influenced by various cell death pathways, notably autophagy and ferroptosis. Recent studies have increasingly highlighted the interplay between autophagy and ferroptosis, a process likely driven by the accumulation of reactive oxygen species (ROS). Post-IS, either the inhibition of autophagy or its excessive activation can escalate ROS levels. Concurrently, the interaction between ROS and lipids during ferroptosis further augments ROS accumulation. Elevated ROS levels can provoke endoplasmic reticulum stress-induced autophagy and, in conjunction with free iron (Fe^{2+}), can trigger ferroptosis. Moreover, ROS contribute to protein and lipid oxidation, endothelial dysfunction, and an inflammatory response, all of which mediate secondary brain injury following IS. This review succinctly explores the mechanisms of ROS-mediated crosstalk between autophagy and ferroptosis and the detrimental impact of increased ROS on IS. It also offers novel perspectives for IS treatment strategies.

1. Introduction

Ischemic stroke (IS), a leading cause of chronic disability and mortality, is categorized into ischemic and hemorrhagic types [1], with over 80% being ischemic [2]. Annually, IS affects approximately 9.6 million people globally. It impacts individuals of various ages, with younger brains typically exhibiting a stronger recovery potential [3]. Consequently, the incidence of IS is predicted to rise in parallel with the aging population. Stroke survivors often experience a range of sequelae, including motor, sensory, speech, mental, and cognitive impairments. These sequelae not only severely affect the quality of life of patients but also place a significant economic burden on both the state and society [4]. Common risk factors for IS include hypertension, hyperlipidemia, hyperglycemia, and smoking [5]. Most cases of IS result from localized disruptions in cerebral blood supply, leading to insufficient oxygenation and subsequent ischemic-hypoxic lesion necrosis [6]. Rapid tissue damage occurs when cerebral blood flow is substantially reduced [7]. The core area of the infarction suffers irreversible damage, surrounded by an ischemic penumbra, a limbic zone susceptible to salvage through hemodynamic and molecular interventions within 3 h of ischemia onset. The ischemic core expands, and the penumbra vanishes within 3–6 h [8]. Current IS treatment strategies focus on reestablishing blood flow, specifically through thrombolytic therapy within a 3–4.5-h window to salvage reversible ischemic injury [9]. However, this narrow therapeutic window and

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ischemia-reperfusion injury often lead to poor prognosis in IS patients [10]. Once this window is missed, damaged neurons cannot be recovered. Hypoxia in the ischemic core reduces adenosine triphosphate (ATP) production, leading to intracellular calcium concentration increase due to Na^+/K^+ pump and plasma membrane Ca^{2+}/ATP pump failure [11]. This calcium overload in neurons triggers cell death pathways, including autophagy and ferroptosis. Additionally, reperfusion can aggravate tissue damage, a phenomenon known as cerebral ischemia-reperfusion injury, involving pathophysiological processes like increased oxygen free radicals, intracellular calcium overload, and inflammatory response overactivation [12]. Thus, exploring effective IS treatment strategies is crucial for improving patient symptoms and prognosis.

Autophagy, a process vital for maintaining intracellular homeostasis by degrading damaged organelles, plays a contentious role in IS, linked to the adaptive capacity of cells. Post-ischemia and hypoxia, adenosine monophosphate (AMP)-activated protein kinase (AMPK) gets activated to enhance autophagy, while the mechanistic target of rapamycin complex 1 (mTORC1) pathway, an autophagy inhibitor, is impaired, thereby inducing autophagy [13]. Reduced autophagy leads to persistent microglial activation, influencing NLRP3 inflammatory vesicles, which release various neurotoxic inflammatory cytokines [14]. In both the mouse cerebral ischemia model and oxygen and glucose deprivation (OGD)-treated neuron model, autophagy has shown protective effects by inhibiting neuronal apoptosis [15,16]. This suggests that inducing autophagy might be a viable therapeutic strategy for IS, offering neuronal protection, reducing brain damage, and benefiting ischemic peripheral brain tissue. However, excessive autophagy can lead to neuronal cell death and motor defects [17], indicating that balanced autophagy is critical in IS treatment, where both inhibition and excessive autophagy can be detrimental.

Iron plays a pivotal role in numerous physiological processes in the body, and even minor disruptions in iron homeostasis can

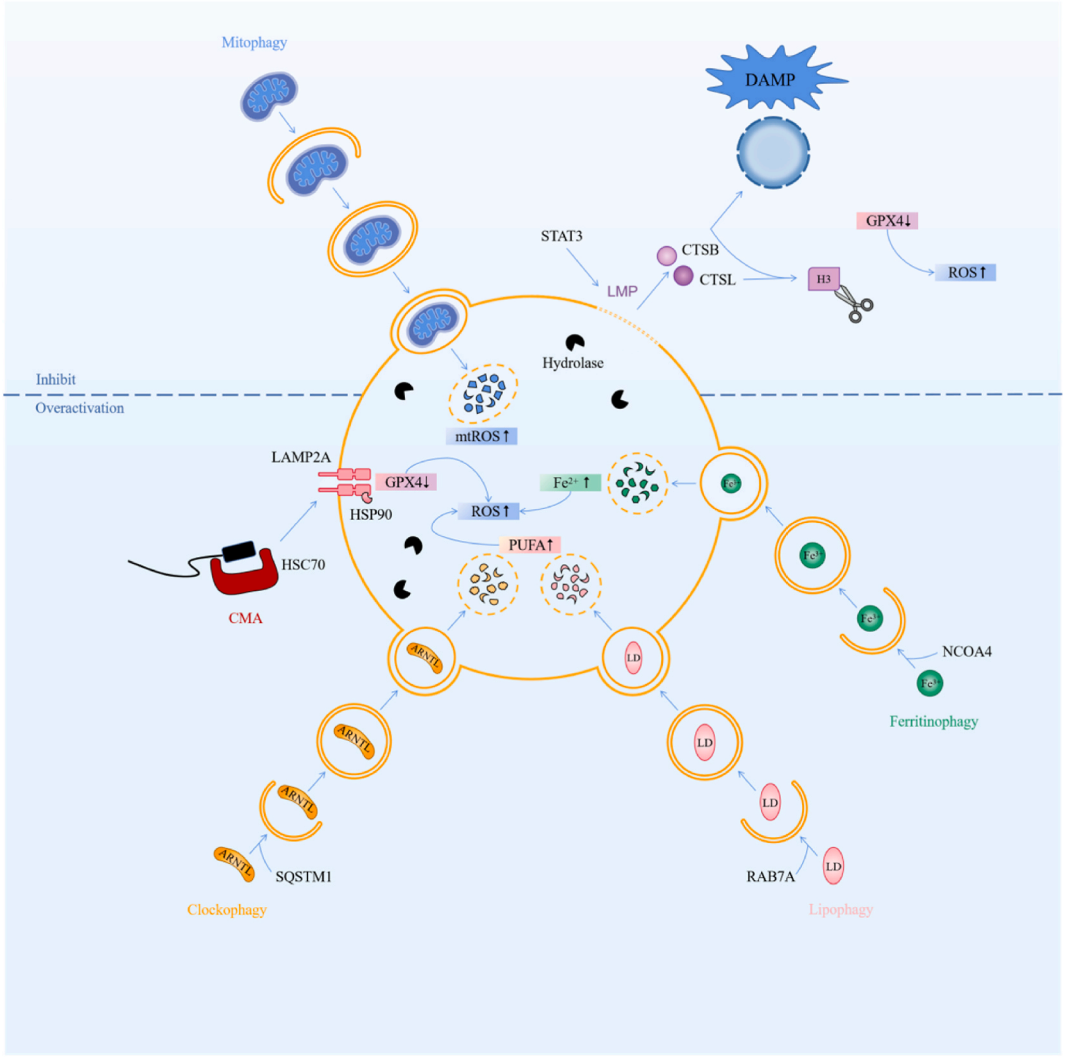

Fig. 1. Mechanisms of Autophagy-Mediated Increase in ROS. Following IS, both inhibition and overactivation of autophagy contribute to the accumulation of excess ROS. Inhibited autophagy leads to mitochondrial dysfunction and lysosomal damage, while overactivation of autophagy through ferritinophagy, lipophagy, clockophagy, and CMA induces increased ROS through diverse.

significantly impact organ function, particularly in the brain. Brain tissue, rich in iron, is crucial for synaptic development, myelin formation, neurotransmitter synthesis, and oxidative metabolism of nerve cells [18]. Iron is essential for the growth and conduction of the nervous system. The brain, being a highly metabolically active organ, is particularly sensitive to variations in iron levels. Both deficiency and excess of iron can disrupt neurophysiological mechanisms. Insufficient iron levels can lead to cognitive impairments in severe cases, while excess iron is linked to various neurological disorders, including IS [19]. Ferroptosis, an iron-dependent cell death pathway discovered in 2012, is characterized by excessive Fe^{2+} and ROS accumulation [20]. It is implicated in numerous neurological disorders, including IS. Post-cerebral ischemia, increased lipid peroxidation and reduced glutathione (GSH) levels promote ferroptosis, leading to neuronal death [21]. Inhibiting ferroptosis can reverse ischemic damage and lessen ischemia-reperfusion injury [22], showing significant potential in IS treatment.

The brain's vulnerability to ROS damage is heightened due to its cholesterol and polyunsaturated fatty acids, which are easily peroxidized and possess low antioxidant capacity [23]. In IS, there exists a crosstalk between autophagy and ferroptosis, largely accountable for the rise in ROS levels, with excessive ROS further inducing both autophagy and ferroptosis. Excess ROS can also lead to other pathological changes, adversely affecting IS prognosis. Research has demonstrated that simultaneously inhibiting apoptotic and necrotic cell death pathways can significantly reduce neurological damage in IS compared to inhibiting a single pathway [24] Another study suggests that a dual-targeted therapy focusing on mitochondrial autophagy and inflammation inhibition might be more effective [14]. Thus, it's plausible that intervening in both autophagy and ferroptosis could further reduce harmful ROS accumulation, thereby mitigating damage caused by IS and ischemia-reperfusion. This review briefly outlines the crosstalk mechanisms between autophagy and ferroptosis in IS through ROS generation and the detrimental effects of increased ROS on IS.

2. Increased autophagy-mediated ROS following IS

Post-cerebral ischemia, autophagy is activated in various brain cell types, including neurons, glial cells, and cerebral microvascular cells [16]. It is well known that autophagy can play a dual role in IS. Moderate activation of autophagy can make neuron cells survive, while excessive autophagy will trigger neuron death. Autophagy offers neuroprotection by inhibiting cell apoptosis and promoting mitochondrial degradation. Both in vivo and in vitro studies have shown that during neuronal ischemia, autophagy is induced via the AMPK-mTOR pathway, playing a protective role [25]. Conversely, sustained induction beyond cellular adaptive capacity turns excessive autophagy harmful, either by triggering neuronal cell death mechanisms or by increasing the permeability of toxic substances across the blood-brain barrier (BBB) [2]. Whether autophagy exerts beneficial or detrimental effects depends on the rate of induction and the duration of activation. Inhibition or overactivation of autophagy leads to mitochondrial dysfunction or lysosome impairment, respectively, either which results in the accumulation of excess ROS. Furthermore, the activation of other autophagy pathways like ferritinophagy, lipophagy, circadian (biological clock) autophagy, and molecular chaperone-mediated autophagy (CMA) can also contribute to increased ROS levels [26] (Fig. 1).

2.1. Inhibition of autophagy after IS leads to increased ROS

Mitochondria, the cell's energy-supplying organelles, have a dual membrane structure. The inner mitochondrial membrane houses the electron transport chains, comprising Complexes I, II, III, and IV. Electrons from NADH and FADH2 are transferred to Complex I and II, respectively, then relayed to Complex III via coenzyme Q, to Complex IV via cytochrome C (Cyt C), and ultimately to oxygen [27]. This process, generating ATP and ROS, is known as oxidative phosphorylation or respiration. When oxygen receives four electrons, it is converted into water, effectively neutralizing the electrons. However, incomplete electron reception by oxygen leads to the formation of ROS. Depending on the electrons received, various ROS forms such as oxygen-negative ions (O^{2-}), hydrogen peroxide (H_2O_2), or hydroxyl radicals (OH^-) can be produced, all highly reactive due to their unpaired electrons.

Post-IS, selective mitochondrial autophagy is activated to identify and transport damaged mitochondria to lysosomes for degradation, thereby maintaining mitochondrial homeostasis crucial for neuronal survival. This activation of mitochondrial autophagy has been shown to protect mitochondria from damage during cerebral ischemia, underscoring its vital role in regulating mitochondrial dysfunction [26]. PINK1/Parkin-mediated autophagy is linked to mitochondrial dynamics and motility. PINK1 and Parkin collaborate to detect and facilitate the removal of impaired mitochondria. Healthy mitochondria eliminate PINK1, but when mitochondria are damaged or depolarized, the loss of membrane potential halts PINK1 degradation, allowing Parkin recruitment to the mitochondrial surface. Parkin then polyubiquitinates outer membrane proteins [28,29], signaling autophagic mechanisms to envelop the damaged mitochondria in autophagic vesicles, thereby preventing oxidative stress damage [30]. Mitochondrial autophagy may also involve PARK2 and BNIP3L. PARK2 localizes to mitochondria following OGD and interacts with other autophagy-related proteins, SQSTM1 and Microtubule-associated protein light chain 3 (LC3), to trigger mitochondrial autophagy. Silencing of PARK2 exacerbates cell death and hinders mitochondrial autophagy [31]. BNIP3L-mediated mitochondrial autophagy can compensate for reduced PARK2 levels, preventing ischemic brain injury. Thus, BNIP3L and PARK2 independently induce mitochondrial autophagy in IS [32].

After IS, mitochondrial autophagy may be inhibited due to decreased levels of PINK1, Parkin, and BNIP3L [33,34]. As a result, damaged mitochondria accumulate and become dysfunctional, producing large quantities of harmful ROS, a major pathological factor in cerebral ischemia and a potential target for therapeutic intervention in IS. Mitochondria possess an array of ROS scavenging systems that collectively regulate oxidative stress caused by mitochondrial ROS [35]. These systems include superoxide dismutases (SODs), which convert highly reactive superoxide free radicals into hydrogen peroxide. This hydrogen peroxide is then further detoxified by catalase, GSH-PX, and PRX/Trx systems. SOD1, SOD2, and SOD3 isoforms of SODs, located in the cytoplasmic, mitochondrial matrix, and extracellular matrix respectively, play key roles in mitochondrial localization and activity control for mitochondrial ROS

clearance. SOD2's activity can be modulated by post-translational acetylation, with Sirt3-mediated deacetylation promoting ROS clearance [36]. Glutathione peroxidases 1 (GPX1) and glutathione peroxidase 4 (GPX4), localized in mitochondria, use reduced glutathione (GSH) to convert hydrogen peroxide into water. Additionally, PRX3 and PRX5 from the PRX peroxidase family are identified in mitochondria, where PRX3 is located in the mitochondrial matrix, and PRX5 in mitochondria, peroxisomes, and cytoplasmic lysosomes. PRX is oxidized during hydrogen peroxide detoxification and simultaneously reduced via Trxs, with Trx2 also located in mitochondria, known to restrict mitochondrial ROS production [37]. According to this evidence, it can be inferred that mitochondrial dysfunction leads to the inability of SOD1, SOD2, GPX1, GPX4, PRX3, PRX5, and Trx2 to clear ROS, and the normal state of equilibrium is disrupted, generating large amounts of ROS.

Mitochondrial dysfunction disrupts the normal equilibrium of these systems, leading to an overproduction of ROS. An imbalance between ROS generation and clearance can cause oxidative stress, impairing mitochondrial respiratory chain function, altering membrane permeability and calcium homeostasis, and weakening mitochondrial DNA and defense systems [38]. The mitochondrial permeability transition pore (MPTP) is highly sensitive to ROS and Ca^{2+} overload, suggesting an amplification loop exacerbated by impaired Ca^{2+} or ROS signaling, triggering both MPTP activation and mitochondrial outer membrane permeabilization. This loop intensifies ROS-induced ROS release or Ca^{2+}-induced Ca^{2+} release, propagating throughout the mitochondria [39]. Subsequently, mitochondria swell and their outer membrane ruptures, releasing calcium ions into the cytoplasm and leading to a vicious cycle of increased Ca^{2+} and ROS production, exacerbating mitochondrial dysfunction [27].

Transcription factor EB (TFEB) has been found to rescue autophagy-lysosome pathway (ALP) dysfunction and alleviate permanent cerebral ischemic injury. Activation of TFEB leads to its nuclear translocation, enhancing the expression of lysosomal proteins and SODs, ultimately reducing ROS [40]. TFEB activity is regulated by PPP3/calcium-regulated neurophosphatase and mTOR, with activation of PPP3 leading to TFEB nuclear translocation, and inactivation of mTOR promoting TFEB movement from the cytoplasm to the nucleus. In vivo studies have shown that post-cerebral ischemia, TFEB's nuclear translocation, and transcriptional activation occur primarily in neurons, with the increase in total TFEB protein possibly due to self-transcription positive feedback, contributing to ALP dysfunction. Other MIT/TFE3 family proteins, like MITF, TFEC, and TFE3 (especially TFE3), also undergo nuclear translocation after cerebral ischemia, but their roles in regulating ALP function remain to be clarified [41].

2.2. Lysosomal damage leads to increased ROS

The signal transducer and activator of transcription 3 (STAT3) is a potential transcription factor [42]. Activated through tyrosine phosphorylation, STAT3 undergoes dimerization and translocates to the nucleus to transcribe target genes. There is mounting evidence indicating STAT3's involvement in autophagy regulation. Specifically, STAT3 transcriptionally enhances the expression of cathepsin B (CTSB) and cathepsin L (CTSL) [43]. The upregulation of CTSB and CTSL leads to cell death via lysosomal membrane permeabilization (LMP) and the subsequent release of lysosomal contents. Two potential mechanisms are identified for mediating GPX4 degradation through CTSB upregulation [44]. As a result, GPX4 expression decreases, diminishing the ability to scavenge lipid ROS and thereby increasing ROS levels. In the first scenario, CTSB translocates from the lysosome to the nucleus, where it accumulates, mediates DNA damage, and releases nuclear damage-associated molecular patterns (DAMPs). This activates the STING1-dependent DNA sensor pathway, leading to GPX4 degradation [45]. Alternatively, CTSB and CTSL are implicated in histone H3 cleavage and the regulation of histone modifications. CTSL cleaves H3 more efficiently, with cleavage products detectable within 30 min. In contrast, CTSB cleavage products appear after 2 h, suggesting an earlier action of CTSL. The cleavage of H3 at various amino acid sites promotes chromosome segregation and subsequently regulates the GPX4 pathway, mediating oxidative damage [46,47]. Moreover, deficiency in prosaposin (PSAP), encoding a lysosomal protein, disrupts lysosomal lipid metabolism, contributing to lipofuscin formation. This accumulation includes reactive Fe^{2+}, producing ROS through the Fenton reaction [48].

2.3. Overactivation of autophagy after IS leads to increased ROS

Autophagy, the process of using lysosomes to degrade damaged organelles and misfolded proteins, plays a vital role in maintaining normal cellular metabolism [49]. It is categorized into three types based on the delivery methods: macroautophagy, microautophagy, and chaperone-mediated autophagy [50]. Autophagy initiation involves the anchoring of the ATG protein and ULK1 protein complex to the preautophagosomal structure. Preautophagosome nucleation, involving the ATG complex and LC3 protein system, leads to the formation of a membrane structure that gradually envelops the damaged organelle, creating a closed bilayer membrane structure known as the mature autophagosome. These mature autophagosomes are transported to the perinuclear region to fuse with lysosomes, forming autophagic-lysosomal structures. This allows lysosomal contents to enter the autophagosome, degrading the enclosed substances and breaking down macromolecules for cellular reuse [51].

Impairment in any of the components of iron uptake, storage, utilization, distribution, and export can lead to the accumulation of Fe^{2+}. Nuclear receptor coactivator 4 (NCOA4) mediates ferritin transfer to lysosomes for degradation, regulating intracellular iron content. While moderate ferritinophagy maintains stable iron levels, excessive ferritinophagy releases substantial amounts of Fe^{2+}. Additionally, ferroportin (SLC40A1), responsible for iron transport out of the cell, when degraded by autophagy, can increase intracellular Fe^{2+} levels [52]. Accumulated Fe2+ undergoes further oxidation to iron (Fe3+) via the Fenton reaction, rapidly converting H2O2 into highly reactive hydroxyl radicals and initiating an oxidation cycle [53,54].

Increasing lipid storage can protect cells from polyunsaturated fatty acids (PUFA)-induced lipotoxicity [55], while lipophagy and clockophagy upregulate PUFA by reducing lipid storage, inducing lipid peroxidation and increasing ROS accumulation. RAB7A-mediated autophagic degradation of intracellular lipid droplets (LDs) elevates intracellular PUFA levels, inducing lipid release

and subsequent lipid peroxidation. PUFA content positively correlates with lipid peroxidation degree [56]. Clockophagy-mediated ARNTL degradation, dependent on SQSTM1/P62, increases EGLN2 expression, promoting lipid peroxidation by inhibiting HIF1A-dependent fatty acid uptake and lipid storage [57,58]. It has been suggested that genetic or pharmacological inhibition of autophagy can prevent cells from accumulating lethal amounts of lipid peroxides. Autophagy inhibitors BafA1 and CQ significantly reduced total ROS. Similarly, the knockout of ATG3 and ATG13 in autophagy-deficient cells resulted in a significant reduction in total ROS [59]. Autophagy, in turn, promotes ROS accumulation, which is associated with erastin-induced iron accumulation. Notably, although inhibition of autophagy was able to reduce erastin-induced cellular ROS, erastin blocked the import of cytosine, thereby depleting a component of GSH synthesis, at which point autophagy inhibition or any other downstream intervention failed to prevent erastin-induced GSH depletion. Subsequent inactivation of GPX4 and uncontrolled nicotinamide adenine dinucleotide phosphate (NADPH)-dependent lipid peroxidation both lead to ROS accumulation [60].

In addition to Fe^{2+} and PUFA accumulation, CMA also contributes to ROS accumulation. CMA involves the recognition of soluble protein substrates with KFERQ sequences via heat shock protein 70 (HSC70) [61]. The HSC70-substrate complex binds to lysosomal-associated membrane protein 2A (LAMP2A) on the lysosomal membrane, transporting the substrate into the lysosomal cavity for degradation. Heat shock protein 90 (HSP90) can increase LAMP2A levels during this process [26,62], subsequently leading to GPX4 inactivation and further ROS accumulation [63].

3. Interaction between ROS and lipids in ferroptosis after IS

The brain's susceptibility to ROS damage aligns with the three critical elements of ferroptosis. Firstly, neuronal and glial cell membranes are abundant in cholesterol and unsaturated fatty acids, making them prone to oxygen radical attacks. Secondly, high iron content in brain tissue, particularly Fe2+, acts as a potent catalyst in oxygen radical reactions during brain injury. Lastly, the brain's relatively low levels of antioxidant enzymes, including SODs and GPX, translate to a diminished capacity for scavenging free radicals and harmful substances [64]. Following IS, the BBB is compromised, allowing Fe^{2+} and ferritin to infiltrate the brain parenchyma. The oxidation of Fe^{2+} and H_2O_2, known as the Fenton reaction [26], generates highly reactive hydroxyl radicals that promote oxidative damage to proteins and lipids, ultimately leading to ferroptosis [65]. A hallmark of ferroptosis is the accumulation of ROS, which oxidize PUFA in lipid membranes, producing toxic phospholipid hydroperoxides (PLOOH). This oxidation process also generates new lipid radicals [66]. These lipid radicals can extract hydrogen from neighboring PUFA side chains through chain reactions or branching, leading to further oxidation and continuous generation of PLOOH. This process perpetuates the chain reaction of lipid peroxidation, amplifying the effects of ROS. This propagation phase can recur multiple times, and the initial event triggering lipid peroxidation can be magnified as long as there is a supply of oxygen and unoxidized PUFA [67,68]. In summary, ferroptosis is characterized by the build-up of intracellular lipid reactive oxygen species.

4. Excess ROS mediates secondary brain injury after IS

Under normal physiological conditions, elevated levels of reactive oxygen species (ROS) can stimulate the synthesis of various growth factors, such as vascular endothelial growth factor (VEGF), placental growth factor (PGF), and angiopoietin 1 (Ang 1) and angiopoietin 2 (Ang 2). These factors play crucial roles in neovascularization, including the recruitment of stem cells from the bone marrow. Studies investigating the protective effects of hyperbaric oxygen therapy (HBOT) during the perioperative period have demonstrated that ROS can positively influence various growth factors involved in angiogenesis. However, this stimulatory effect appears to be transient, as indicated by the increase in growth factors only after the initial HBOT treatment [69]. When ROS levels become excessively high, they may lead to oxidative damage, cell death, and an overactive inflammatory response, which are detrimental in the context of IS. The accumulation of ROS during thrombolytic therapy following cerebral ischemia can further exacerbate ischemia-reperfusion injury [70]. Current research highlights that neuronal death and the secondary inflammatory response following IS are critical factors in the development of brain injury. The delicate balance between beneficial and harmful effects of ROS underscores the complexity of their role in both physiological and pathological processes, particularly in the aftermath of IS.

4.1. Excess ROS induces endoplasmic reticulum stress to induce autophagy

Under normal conditions, the endoplasmic reticulum (ER) is crucial for protein synthesis and processing, lipid secretion, and metabolic processes [71]. ER stress activates the unfolded protein response (UPR) to restore protein homeostasis. However, if cells fail to reestablish homeostasis, cell death ensues [72]. The application of MnTMPYP, a mimetic of the ROS inhibitor MnSOD, inhibits ROS increase in ER stress (ERS), thereby delaying UPR activation. This suggests that ROS is an upstream signal triggering ERS to initiate UPR. The UPR activates three stress sensors on the ER membrane: membrane-anchored activating transcription factor 6 (ATF6), serine/threonine-protein kinase/endoribonuclease inositol-requiring enzyme 1 α (IRE1), and pancreatic ER kinase (PKR)-like endoplasmic reticulum kinase (PERK) [73]. IRE1, a bifunctional serine/threonine kinase and endoribonuclease, induces autophagy via the autophagy-JNK signaling pathway [74], mediating Bcl-2 phosphorylation and upregulating the autophagy gene Beclin-1 [75]. Additionally, GPX4 depletion during ferroptosis, leading to lipid peroxidation, can inhibit STING activity by producing 4-hydroxynonenal (4-HNE) [76]. The TMEM173/STING-dependent DNA sensor pathway, activated by 8-OHG release, also triggers a UPR response [77]. STING1 on the endoplasmic reticulum acts as a key junction protein that mediates intracellular DNA activation of the innate immune response in response to cGMP stimulation, thereby exerting an immune defense role. It was reported in early 2009 that

activated STING was first found to co-localize not only with ULK1, ATG5, or ATG14L but also with autophagy protein, LC3, and autophagy-associated gene 9A (ATG9A), verifying a close interrelation between STING activation and autophagy induction [76]. Endoplasmic reticulum stress induced by STING activation initiates UPR and ultimately induces autophagy through the activation of IRE1 [73]. Another stress sensor on the endoplasmic reticulum membrane, PERK, can also be activated through the UPR response and activated PERK leads to phosphorylation of the translation initiation factor 2α (eIF2α), which is able to induce the key transcription factor ATF4. ATF4 further promotes autophagy by regulating ATG5 through the activation of C/EBP homologous protein (CHOP) or directly regulating ATG12 [78]. In addition, activation of the stress sensor ATF6 can similarly promote autophagy through CHOP [79].

In addition to direct regulation of autophagy through UPR pathway-related proteins, ROS can also indirectly regulate autophagy by altering the concentration of calcium ions in the cytoplasm. ER is the main intracellular Ca^{2+} reservoir, and the presence of ROS affects the channel function of ER, which in turn affects Ca^{2+} homeostasis. Ca^{2+} leakage into the cytosol can induce autophagy either by activating an AMPK-dependent pathway through calcium/calmodulin-dependent kinase β (CaMKKβ) to inhibit mTOR activity or by activating death-associated protein kinase (DAPK) to upregulate the autophagy gene Beclin-1 or by activating protein kinase C (PKC) to upregulate LC3 through an mTOR-independent pathway [80] (Fig. 2).

Moderate autophagy maintains neuronal homeostasis by removing protein aggregates and damaged mitochondria. However, increased ROS can lead to excessive autophagy, enhancing the degradation of essential components and ultimately causing cell death [2]. An increase in ROS induces autophagy either by triggering the UPR response or by affecting Ca^{2+} homeostasis. Excessive autophagy negatively impacts IS. For example, occludin (OCLN) is integral to the stability of intracellular tight junctions (TJs) and maintaining BBB integrity [2]. Excessive autophagy promotes OCLN degradation [81], increasing BBB permeability to toxic substances [82]. Structural disruption and increased permeability of TJs are key features of BBB dysfunction [83], leading to detrimental outcomes like increased brain water content and tissue swelling [84], nerve damage, hemorrhagic transformation, and elevated mortality post-IS [85].

4.2. Excess ROS leads to ferroptosis

Ferroptosis, a form of cell death, is primarily induced by three key elements: iron overload, lipid peroxidation, and the inactivation of GPX4. Central to the process of ferroptosis, as the term implies, is iron. Excess iron catalyzes the production of ROS via the Fenton reaction, driving lipid peroxidation. In this process, ROS react with phospholipids containing polyunsaturated fatty acids (PUFA-PL), using Fe^{2+} as a catalyst, to produce harmful lipid peroxides [67]. Two crucial enzymes in the lipid peroxidation pathway, acyl coenzyme A synthetase long-chain family member 4 (ACSL4) and lysophosphatidylcholine acyltransferase 3 (LPCAT3), facilitate this process. ACSL4 catalyzes the binding of long-chain PUFAs to coenzyme A (CoA), and LPCAT3 aids in esterifying and incorporating these into membrane phospholipids [86,87]. Additionally, arachidonate lipoxygenase (ALOX) or cytochrome P450 oxidoreductase

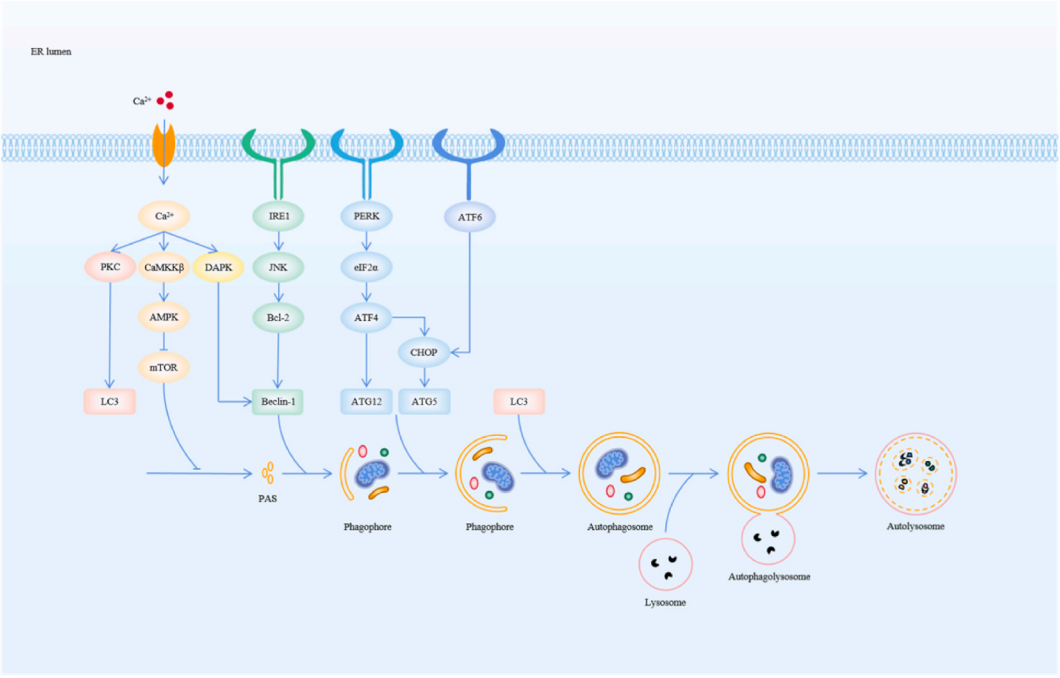

Fig. 2. ROS can directly regulate autophagy through the activation of the three stress sensors IRE1, PERK, and ATF6 on the ER membrane, and indirectly by altering intracytoplasmic calcium ion concentration. Excessive accumulation of ROS induces autophagy by upregulating autophagy-related genes or regulating autophagy-related signaling pathways, as depicted in the figure.

(POR)-mediated enzymatic reactions contribute to the oxidation of PUFA on cell membranes, promoting lipid peroxidation [86,88].

The System Xc-GSH-GPX4 axis is vital in regulating ferroptosis [89]. Cystine is transported into the cell via the dimerization of the two subunits, SLC7A11 and SLC3A2, of the glutamate-cystine transporter system Xc-embedded in the cell membrane surface and is reduced to cysteine, which is then synthesized into GSH [90]. GPX4 uses reduced GSH to neutralize harmful phospholipid hydroperoxides, converting them into non-toxic phospholipids [91]. This process mitigates peroxide toxicity and provides resistance against ferroptosis [92]. During ferroptosis, erastin inhibits System Xc-, leading to cystine uptake disruption, resulting in GSH depletion and GPX4 inactivation [93]. Similarly, the GPX4 inhibitor RSL3 depletes GSH, reducing the ability to scavenge lipid ROS [94], culminating in lipid peroxidation and cell death. Hence, ferroptosis is characterized by erastin-induced cell death accompanied by a rise in ROS [95]. Ferroptosis plays a role in various neurodegenerative diseases, including stroke, brain hemorrhage, traumatic brain injury, and ischemia-reperfusion injury [96,97]. Inhibitors of ferroptosis have shown promising neuroprotective effects, significantly reducing neuronal death mediated by ferroptosis, improving neurological function, and decreasing brain infarct size in mouse models [22,98]. This indicates that ferroptosis intensifies neuronal death following IS [21,22], contributing to secondary brain damage.

4.3. Excess ROS mediate other secondary brain damage after IS

Excess ROS can cause significant cellular damage, including to proteins, lipids, and DNA, ultimately impairing neurological function [99]. First, almost all proteins can be oxidatively damaged by ROS. It has been argued that the main determinant of oxidative protein damage is the resistance of proteins to oxidative damage [100], rather than changes in ROS concentration and duration of action. ROS can mediate protein oxidation both directly and indirectly by attacking lipids and then using hydroxyl radicals and intermediates in their oxidation process [101]. Furthermore, ROS-induced DNA damage can inhibit gene expression, lead to protein misfolding, and affect protein synthesis. Protein misfolding, in turn, increases vulnerability to oxidative damage [100]. Oxidized lipids and proteins play a role in atherogenesis and the progression of atherosclerosis, leading to ischemic complications, including IS.

Endothelial dysfunction (ED) is a key factor in the progression of atherosclerosis and related complications. Endothelial cells produce nitric oxide (NO) to regulate vascular tone by maintaining a balance between vasodilation and contraction [102]. Endothelial nitric oxide synthase (eNOS) catalyzes the generation of NO from L-arginine to L-citrulline, requiring BH4 for stable activity. ROS can degrade BH4, leading to eNOS uncoupling and reduced NO bioavailability, resulting in ED [103]. Elevated levels of asymmetric dimethylarginine (ADMA), an endogenous inhibitor of eNOS, can also contribute to eNOS uncoupling [104]. Excess ROS can decrease the activity of dimethylarginine dimethylaminohydrolase (DDAH), the enzyme metabolizing ADMA, and upregulate protein arginine methyltransferase 1 (PRMT1), which converts L-arginine to ADMA [105–107]. Decreased BH4 levels or increased ADMA levels reduce NO bioavailability, leading to ED. ED contributes to the disruption of vascular endothelium structure and increased permeability, facilitating the entry of macromolecules like LDL into the subendothelial space, where they are oxidized and phagocytosed by macrophages, forming foam cells and accumulating in the blood vessel wall [108]. This process leads to systemic atherosclerosis, causing ischemia in organ tissues, including the brain, and leading to IS and cardiovascular complications [109,110].

Oxidative stress and inflammation often coexist and exacerbate each other in many pathological conditions. ROS can cause morphological and functional changes in glial cells, contributing to neuroinflammation, excitotoxicity, and BBB damage [111]. Under oxidative stress conditions, ROS leads to the dissociation of thioredoxin-interacting proteins (TXNIP) from thioredoxin and binding to NLRP3, the highest expressed in microglia in the NOD receptor (NLR) family. Subsequent activation of NLRP3 inflammatory vesicles and polarization of the microglia phenotype toward the pro-inflammatory type (M1 type) produces interleukin-1beta (IL-1β) [14,112]. Astrocytes can also activate NLRP3 and produce IL-1β [113]. Moreover, large amounts of ROS can activate the NF-κB pathway, increasing the expression of pro-inflammatory cytokines like IL-1β [114]. The extracellular redox potential, influenced by the oxidation of plasma cysteine (Cys) and cystine (CySS), can promote monocyte adhesion to vascular endothelial cells, activating NF-κB and increasing IL-1β expression [115]. Activated phagocytes, such as neutrophils and macrophages, produce large quantities of ROS under pathological inflammatory conditions, increasing local oxidative stress and tissue damage [116,117]. Inflammatory cells also produce soluble mediators that recruit more inflammatory cells to the injury site and activate redox-sensitive signaling pathways like c-Jun N-terminal kinase (JNK), p38 MAP kinase, and transcription factor AP-1 [115,118]. This vicious cycle between oxidative stress and inflammation can continue to affect pathological changes post-IS, impacting long-term neurological function.

5. Conclusion

The prevalence of IS is known to increase with age, while the brain's recovery potential diminishes. This trend can be attributed to long-term lifestyle habits such as hypertension, hyperlipidemia, hyperglycemia, and smoking, which, if not effectively managed, significantly elevate the risk of IS. Aging also leads to a decline in the production of SODs, crucial for maintaining the body's oxidative-antioxidant balance, resulting in the accumulation of ROS [119]. Following an IS event, both autophagy and ferroptosis contribute to an increase in ROS. Excessive ROS, surpassing the clearance capacity of the body's antioxidant mechanisms, further induce autophagy through ERS and collaborate with lipids to trigger ferroptosis. Additionally, ROS can damage proteins and lipids and induce endothelial dysfunction and inflammatory responses, all of which are factors that contribute to the onset and progression of IS. This review underscores the potential of future treatment strategies for IS, highlighting the role of antioxidant therapy in reducing ROS production and mitigating its harmful effects, including local ischemia, ischemia-reperfusion injury, and secondary brain damage. Regular exercise training is beneficial in enhancing the body's antioxidant capacity and increasing the ability to eliminate ROS, thereby preventing or slowing the progression of IS damage [120]. However, it's important to note that excessive exercise can lead to a significant increase in ROS production, emphasizing the need to moderate exercise intensity. Furthermore, antioxidants can neutralize excess free

radicals, effectively controlling ROS levels and offering protection against IS [121]. Understanding the intricate relationship between ROS, autophagy, ferroptosis, and other cellular mechanisms is crucial in developing comprehensive and effective approaches to IS treatment and prevention.

Autophagy and ferroptosis, as two distinct cell death pathways, have unique mechanistic and morphological characteristics and are regulated by different key factors. However, recent research has increasingly highlighted a complex interplay between these two pathways following IS, with oxidative stress playing a central role in this crosstalk. A multitude of studies have focused on the role of autophagy in IS, suggesting that ischemic injury is often associated with disrupted autophagy. After cerebral ischemia, autophagy is activated in various cell types within the brain, including neurons, glial cells, and cerebral microvascular cells. This response is typically characterized by an initial upregulation of autophagy soon after ischemic injury, followed by a subsequent inhibition. Experimental evidence from animal studies has shown that moderate autophagy can decrease neuronal death and exert neuroprotective effects, evidenced by reductions in infarct volume and improvements in motor function. Conversely, both inhibited and excessive autophagy are detrimental, promoting cell death. A comprehensive review in 2022 explored the mechanisms of neuronal cell death after IS, including apoptosis, necroptosis, autophagy, ferroptosis, parthanatos, phagoptosis, and pyroptosis [13]. Building upon this, the current focus on autophagy and ferroptosis after IS is particularly relevant. Different stages of autophagy involve specific related genes, which are essential for its regulation. This regulation can be achieved through the use of inhibitors and inducers or by the knockdown and overexpression of related genes. The mechanism by which autophagy exacerbates brain damage post-IS is linked to pathological processes such as BBB destruction, heightened neuroinflammation, and excessive oxidative stress. Recent studies focusing on autophagy post-IS onset have paid particular attention to microglia and astrocytes [122,123]. Given the crucial roles of glial cells in supporting, nourishing, isolating, protecting, and repairing neurons, dysfunctional microglia and astrocytes can lead to neuronal cell death. This connection underscores the importance of understanding and potentially targeting glial cell function in the context of IS treatment and prevention strategies. Addressing the balance and interaction between autophagy and ferroptosis could offer new avenues for therapeutic interventions in mitigating the impact of IS.

Although ferroptosis is the most recently discovered among various cell death pathways, it has rapidly become a significant focus of both basic and clinical research in the context of IS. Ferroptosis, characterized by iron-dependent lipid peroxidation, plays a crucial role in the pathology of IS. In animal models, such as the mouse middle cerebral artery occlusion (MACO) model, significant iron deposition has been observed in the ischemic penumbra, leading to neuronal ferroptosis [22]. This finding is corroborated by clinical evidence: Magnetic Resonance Imaging (MRI) studies in patients with IS have revealed abnormal iron deposition in the periphery of the infarct zone on the affected side [124]. Further research has directly confirmed elevated iron ion levels in the serum of IS patients [125]. These findings collectively highlight a strong link between iron ion abnormalities and the development of IS. Recent studies, from 2020 onwards, have shown that ACSL4 exacerbates neuroinflammation and neuronal death post-IS by promoting ferroptosis through lipid peroxidation [21,126]. Targeting ACSL4 to inhibit its function can suppress the production of pro-inflammatory cytokines and provide neuroprotective effects. Therefore, the strategic inhibition of ferroptosis is inseparably linked to the regulation of lipid peroxidation. A range of inhibitors that can reduce lipid peroxidation levels in the body shows potential in reducing ferroptosis following IS. Moreover, oxidative stress plays a pivotal role in ischemia/reperfusion (I/R) injury. There is a broad consensus in the research community that inhibiting oxidative stress can effectively attenuate neuronal damage following I/R [127]. This body of research underlines the importance of understanding and targeting oxidative stress and lipid peroxidation in managing and treating IS, particularly in the context of ferroptosis. These insights not only advance our understanding of the pathophysiology of IS but also open up new avenues for therapeutic interventions targeting these specific cell death pathways.

The interplay between autophagy and ferroptosis undoubtedly contributes to poor prognosis in IS, and exploring combined interventions targeting both pathways to reduce ROS accumulation could potentially minimize IS-related damage. Beyond the autophagy-ferroptosis crosstalk, the interactions and mechanisms of other cell death pathways in this context remain an area ripe for research and could offer promising treatment avenues for IS. Autophagy is monitored by assessing the conversion of LC3 from its soluble form (LC3-I) to the lipid-bound form (LC3-II) [128], as well as the degradation of P62/SQSTM1 in autophagic lysosomes [129]. Additionally, the change in lysosomal acidity is a useful indicator of autophagy progression [130]. Given the complexity of autophagy, a comprehensive evaluation typically requires multiple assays to provide an accurate and realistic analysis. The therapeutic window for IS has traditionally focused on thrombolysis within 3–6 h post-ischemia, after which the salvageable penumbra diminishes [131]. Similarly, the optimal timing for autophagy intervention in IS is an open question. Research indicates dynamic changes in lysosomal function post-cerebral ischemia, with a marked decline beginning 24 h post-ischemia, suggesting lysosome rupture in the later stages of IS [41]. Thus, identifying the optimal timing for autophagy intervention is crucial. While moderate autophagy has been shown to protect brain tissue post-ischemia and hypoxia, excessive autophagy can lead to cell death. Determining the precise level of autophagy for maximal protection requires further investigation. All forms of autophagy, including macroautophagy, microautophagy, and chaperone-mediated autophagy, rely on lysosomal hydrolytic enzymes for degradation. Therefore, lysosomal damage can significantly impact autophagy levels. Research into how to mitigate autophagy-induced damage by protecting lysosomal function is a valuable direction for long-term investigation. In summary, the field of IS treatment presents substantial opportunities for further exploration. Understanding the intricate balance and regulation of cell death pathways, particularly autophagy and ferroptosis, as well as the role of lysosomal function, could lead to more effective therapeutic strategies for IS.

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Xing-Yu Zhang: Writing – review & editing, Writing – original draft, Visualization, Conceptualization. **Ping-Ping Han:** Supervision, Project administration, Investigation. **Yi-Ning Zhao:** Project administration, Investigation. **Xin-Ya Shen:** Visualization, Supervision, Conceptualization. **Xia Bi:** Writing – review & editing, Supervision, Conceptualization.

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The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Abbreviations

IS	Ischemic stroke
ROS	Reactive oxygen species
ATP	Adenosine triphosphate
AMP	Adenosine monophosphate
AMPK	Adenosine monophosphate-activated protein kinase
mTORC1	Mechanistic target of rapamycin complex 1
OGD	Oxygen and glucose deprivation
GSH	Glutathione
BBB	Blood-brain barrier
Cyt C	Cytochrome C
O^2	Oxygen-negative ions
H_2O_2	Hydrogen peroxide
OH	Hydroxyl radicals
LC3	Light chain 3
SODs	Superoxide dismutases
GPX	Glutathione peroxidase
MPTP	Mitochondrial permeability transition pore
TFEB	Transcription factor EB
ALP	Autophagy-lysosome pathway
STAT3	Signal transducer and activator of transcription 3
GPX1	Glutathione peroxidase 1
GPX4	Glutathione peroxidase 4

CTSB	Cathepsin B
CTSL	Cathepsin L
LMP	Lysosomal membrane permeabilization
DAMP	Damage-associated molecular patterns
STING1	Stimulator of interferon response CGAMP interactor 1
PSAP	Prosaposin
PUFA	Polyunsaturated fatty acids
LDs	lipid droplets
NADPH	Nicotinamide adenine dinucleotide phosphate
CMA	Chaperon-mediated autophagy
HSC70	Heat shock protein 70
LAMP2A	Lysosomal-associated membrane protein 2A
HSP90	Heat shock protein 90
PLOOH	Phospholipid hydroperoxides
VEGF	Vascular endothelial growth factor
PGF	Placental growth factor
Ang 1	Angiopoietin 1
Ang 2	Angiopoietin 1
HBOT	Hyperbaric oxygen therapy
ER	Endoplasmic reticulum
UPR	Unfolded protein response
ERS	Endoplasmic reticulum stress
ATF6	Activating transcription factor 6
IRE1	Inositol-requiring enzyme 1 α
PERK	Protein Kinase R-like endoplasmic reticulum kinase
4-HNE	4-hydroxynonenal
eIF2α	Translation initiation factor 2α
CHOP	C/EBP homologous protein
CaMKKβ	Calcium/calmodulin-dependent kinase β
DAPK	Death-associated protein kinase
PKC	Protein kinase C
OCLN	Occluding
TJs	Tight junctions
PUFA-PL	Phospholipids containing polyunsaturated fatty acids
ACSL4	Acyl coenzyme A synthetase long chain family member 4
LPCAT3	Lysophosphatidylcholine acyltransferase 3
CoA	Coenzyme A
ALOX	Arachidonate lipoxygenase
POR	Cytochrome P450 oxidoreductase
NO	Nitric oxide
eNOS	Endothelial nitric oxide synthase
ED	Endothelial dysfunction
ADMA	Asymmetric dimethyl arginine
DDAH	Dimethylarginine dimethylaminohydrolase
PRMT1	Protein arginine methyltransferase 1
TXNIP	Thioredoxin-interacting proteins
NLR	NOD receptor
IL-1β	interleukin-1beta
Cys	Cysteine
CySS	Cystine
JNK	c-Jun N-terminal kinase
ASCL4	Acyl-CoA synthetase long-chain family member 4
MCAO	Middle cerebral artery occlusion
MRI	Magnetic Resonance Imaging
I/R	ischemia/reperfusion

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