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Review Article

Targeting RNS/caveolin-1/MMP signaling cascades to protect against cerebral ischemia-reperfusion injuries: potential application for drug discovery

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Abstract

Reactive nitrogen species (RNS) play important roles in mediating cerebral ischemia-reperfusion injury. RNS activate multiple signaling pathways and participate in different cellular events in cerebral ischemia-reperfusion injury. Recent studies have indicated that caveolin-1 and matrix metalloproteinase (MMP) are important signaling molecules in the pathological process of ischemic brain injury. During cerebral ischemia-reperfusion, the production of nitric oxide (NO) and peroxynitrite ($ONOO^-$), two representative RNS, down-regulates the expression of caveolin-1 (Cav-1) and, in turn, further activates nitric oxide synthase (NOS) to promote RNS generation. The increased RNS further induce MMP activation and mediate disruption of the blood-brain barrier (BBB), aggravating the brain damage in cerebral ischemia-reperfusion injury. Therefore, the feedback interaction among RNS/Cav-1/MMPs provides an amplified mechanism for aggravating ischemic brain damage during cerebral ischemia-reperfusion injury. Targeting the RNS/Cav-1/MMP pathway could be a promising therapeutic strategy for protecting against cerebral ischemia-reperfusion injury. In this mini-review article, we highlight the important role of the RNS/Cav-1/MMP signaling cascades in ischemic stroke injury and review the current progress of studies seeking therapeutic compounds targeting the RNS/Cav-1/MMP signaling cascades to attenuate cerebral ischemia-reperfusion injury. Several representative natural compounds, including calycosin-7-O-β-D-glucoside, baicalin, *Momordica charantia* polysaccharide (MCP), chlorogenic acid, lutein and lycopene, have shown potential for targeting the RNS/Cav-1/MMP signaling pathway to protect the brain in ischemic stroke. Therefore, the RNS/Cav-1/MMP pathway is an important therapeutic target in ischemic stroke treatment.

Keywords: Ischemic stroke; caveolin-1; reactive nitrogen species (RNS); MMPs; natural compound

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Pathophysiology of acute ischemic stroke

Stroke is one of the most prevalent diseases with high mortality and disability all over the world[1]. Ischemic stroke and hemorrhage stroke are two major subtypes, among which ischemic stroke accounts for more than 80 percent of stroke incidences[2]. Currently, tissue plasminogen activator (t-PA) is the only FDA approved drug for ischemic stroke, and its efficacy is limited by the restrictive golden time window of 4.5 h[3] with the potential risk of hemorrhagic transformation when given beyond this time window[4, 5]. The development of novel therapeutic agents has become timely and important for improving the outcome of ischemic stroke treatment.

Ischemic stroke involves different pathophysiological cascades, including energy failure, oxidative stress, acidosis, disruption of ion homeostasis, calcium overload, neuronal cell excitotoxicity, inflammation, *etc*[6-11]. In ischemic stroke, the obstruction of blood flow dramatically reduces glucose and oxygen supply in ischemic brain region and triggers "ischemic cascades"[12, 13]. Lack of ATP synthesis with low oxygen supply leads to accumulation of lactate and malfunction of ion pumps, including the Na^+/K^+-ATPase and Ca^{2+}/H-ATPase[14], subsequently inducing membrane depolarization and calcium ion (Ca^{2+}) overload[15]. In the meantime, membrane depolarization causes the release of excitotoxic amino acids, especially leading to glutamate translocation into the extracellular compartment. Glutamate can act to induce neurotoxicity, activate glutamate receptors and promote the influx of Ca^{2+}[16].

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A substantial elevation in intracellular Ca^{2+} activates various calcium-dependent enzymes, including protein kinase C, phospholipase A2, phospholipase C, cyclooxygenase, calcium-dependent nitric oxide synthase (NOS), calpain, proteases and endonucleases, resulting in necrotic and apoptotic cell death[17, 18]. Inflammation is another important process of cell death in ischemic stroke[19]. Ischemic cascades activate resident microglia and astrocytes, together with infiltrated T lymphocytes, neutrophils, and macrophages, subsequently inducing the release of multiple inflammatory factors such as cytokines, chemokines, enzymes and free radicals[19, 20]. Therefore, ischemic stroke is a complicated pathophysiological process involving the activation of regulatory networks in response to stroke.

Free radicals are considered to be important players in ischemic stroke, particularly in cerebral ischemia-reperfusion injury. There are two species of free radicals including reactive oxygen species (ROS) and reactive nitrogen species (RNS). ROS are comprised of superoxide, hydroxyl radical, singlet oxygen, hydrogen peroxide, *etc*. ROS at a low concentration serve as redox signaling molecules to maintain biological functions under physiological conditions, whereas large amounts of ROS produced from ischemic brains exacerbate brain injury through different mechanisms[21, 22]. For example, ROS enhance inflammatory responses by activating adhesion molecules and promoting leucocyte infiltration[23]. ROS induce the release of glutamate and calcium overload[24]. ROS activate inflammation factors, mediate lipid peroxidation, and induce neural cell death, disrupting the integrity of the blood-brain barrier (BBB) and enlarging the infarction volume[22]. As representative antioxidants, edaravone, NXY-059, and allopurinol improved outcomes in acute ischemic stroke patients[25-27]. Hence, free radicals aggravate the brain damage in ischemic stroke, and antioxidants may be beneficial in ischemic stroke treatment.

While ROS-mediated ischemic brain injury has been intensively investigated, the roles of RNS remain relatively unexplored. RNS, including NO and $ONOO^-$, mediate the BBB disruption, infarction enlargement and apoptotic cell death in cerebral ischemia-reperfusion injury[28]. RNS-mediated matrix metalloproteinase (MMP) activation is one of the critical pathological processes in cerebral ischemia-reperfusion injury[29, 30]. MMP-9 has been used as a biomarker for monitoring brain damage and predicting hemorrhagic transformation in thrombolytic treatment for ischemic stroke[31]. N^G-nitro-*L*-arginine methyl ester (*L*-NAME), a nonselective NOS inhibitor, significantly reduced the BBB breakdown and MMP-9 activity in a middle cerebral artery occlusion (MCAO) animal model[32]. In the past decade, we have made great efforts in the exploration of the roles of RNS in cerebral ischemia-reperfusion injury. In this mini-review, we mainly focus on the roles of RNS/caveolin-1/MMP signaling cascades in acute ischemic brain injury. Subsequently, we review the potential natural compounds targeting RNS/caveolin-1/MMP signaling pathways for ameliorating cerebral ischemia-reperfusion injury.

Detection of NO and $ONOO^-$ in cerebral ischemia-reperfusion injury

As representative RNS, NO and $ONOO^-$ are produced at both the ischemia and reperfusion stages in cerebral ischemia-reperfusion injury. A low concentration of NO produced from endothelial nitric oxide synthase (eNOS) has physiological functions, whereas a high concentration of NO produced from inducible NOS (iNOS) and neuronal NOS (nNOS) is detrimental to the ischemic brain[28]. When NO and superoxide (O_2^-) are simultaneously produced in the ischemic brain, they rapidly react with each other to produce $ONOO^-$ at a diffusion-limited rate[28]. By using electron paramagnetic resonance (EPR) spin trapping technology, we monitored the production of NO in a rat MCAO model and found that cerebral ischemia-reperfusion resulted in a biphasic increase in NO production in the ischemic core and penumbra, with first-phase NO production at the ischemic phase and the second-phase increase of NO at the reperfusion stage[33]. Notably, a large amount of superoxide (O_2^-) was generated from neurons and endothelial cells by activating NADPH oxidase[34], xanthine oxidase[35], and cyclooxygenase (COX)[36, 37]. The reaction of O_2^- and NO rapidly forms $ONOO^-$. Peroxynitrite induces protein tyrosine nitration by the addition of a nitro group to the hydroxyl group of the tyrosine residue to form 3-nitrotyrosine (3-NT), a footprint marker for $ONOO^-$ production[38]. The production of NO from iNOS and nNOS appears to be important for $ONOO^-$ formation as iNOS or nNOS knockout mice did not show nitrotyrosine-positive staining[39, 40]. Due to the concerns of the sensitivity and specialty of 3-NT for $ONOO^-$[41], we have made great efforts to develop novel specific and sensitive probes for $ONOO^-$ detection[42-44]. With $ONOO^-$ probes, we directly visualized the $ONOO^-$-induced fluorescence in ischemic brains *in vivo* as well as hypoxic neurons *in vitro*. Our results suggest that targeting $ONOO^-$ could be an important strategy not only for attenuating cerebral ischemia-reperfusion injury[45, 46] but also for reducing hepatic ischemia-reperfusion injury[47].

Roles of peroxynitrite in cerebral ischemia-reperfusion injury

Peroxynitrite has a much higher cytotoxicity than NO and O_2^-. Peroxynitrite has an approximately 400 times higher penetrating capacity across the lipid membrane than superoxide anions[48, 49]. Peroxynitrite penetrates the lipid bilayers of the membrane and induces DNA damage[50], protein nitration[51, 52], and lipid peroxidation[53], as well as enzyme and ion channel inactivation[54, 55]. Peroxynitrite, rather than NO, directly mediated poly(ADP-ribose) synthase (PARS) activation and suppressed cellular viability[56]. The peroxynitrite decomposition catalyst FeTMPyP reduced brain infarct volume, inhibited neuronal cell death in the Cornu Ammonis 1 (CA1) region of the hippocampus and improved functional outcomes[57, 58]. Our recent study showed that FeTMPyP significantly reduced the $ONOO^-$ level in ischemic brains and attenuated neuronal apoptosis[59]. Uric acid, an $ONOO^-$ scavenger, rescued over 70 percent of the ischemic cortex and striatum[60]. In addition to neuronal injury, cerebrovascular injury was also induced by

peroxynitrite. The $ONOO^-$ donor 3-morpholino sydnonimine (SIN-1) further reduced the expression of tight junction protein ZO-1 and exacerbated BBB disruption in a cerebral ischemia-reperfusion animal model[61]. Intravenous administration of FeTMPyP significantly reduced neurovascular injury in a prolonged brain ischemia model[62]. In clinical studies, a high level of uric acid in the blood was correlated with excellent outcomes in stroke patients[63, 64]. A meta-analysis involving 10 studies with 8131 ischemic stroke patients also showed a positive correlation of serum uric acid with good neurological outcomes[65]. In addition, the plasma 3-NT level was positively correlated with the magnitude of the brain injury among ischemic stroke patients[66]. Together, these works indicate that $ONOO^-$ could be an important target for ischemic stroke.

Interaction of RNS and MMPs in ischemic brain injury

MMPs are proteolytic enzymes that are capable of disrupting the extracellular matrix (ECM) to mediate ischemic brain injuries[67, 68]. MMPs have a common configuration that includes a zinc-dependent catalytic site, propeptide cysteine switch and other entities, such as a transmembrane domain, fibronectin-binding site and so on[69]. MMP-9 and MMP-2 are two well-known MMPs that contribute to cerebral ischemia-reperfusion injury. The basal level of MMPs in the adult brain is low, but ischemic insults trigger acute activation of several MMPs[70-72]. Stroke patients have a significantly higher serum level of MMP-2 and MMP-9 than healthy controls[73]. Tissue plasminogen activator (t-PA) treatment further enhanced the serum MMP-9 level[73]. Neutrophils and microvessels are major sources of MMP-9 activation and contribute to hemorrhagic transformation in the presence or absence of t-PA during ischemic stroke[74, 75]. Inhibition of MMPs protected against the sustained loss of tight junction proteins such as claudin-5 and occludin in rodent MCAO models[30, 76, 77]. Broad-spectrum and specific MMP-9 inhibitors notably attenuated hippocampal neuronal damage in a transient global cerebral ischemia model[78, 79]. The MMP-9-neutralizing antibody greatly decreased infarction size in ischemic brain injury[80]. MMP-9 KO mice showed a smaller lesion volume than wild-type mice after cerebral ischemia[81]. These results together indicate that MMP-9 plays an important role in mediating cerebral ischemia-reperfusion injury.

RNS activate MMPs during ischemic brain injury[82]. Peroxynitrite was co-localized with MMP-9 in brain microvessels of the area showing Evans blue leakage, suggesting that $ONOO^-$ may induce MMP-9 activation and contribute to BBB disruption[32]. The nonselective NOS inhibitor *N*(omega)-nitro-*L*-arginine (*L*-NA) reduced the 3-NT level and attenuated MMP-9 expression and EB extravasation during cerebral ischemia-reperfusion[83]. Consistently, S-nitrosoglutathione (GSNO) inhibited MMP-9 activation, up-regulated the expression of tight junction protein ZO-1, and ameliorated BBB leakage in ischemic brains[61]. Intravenous administration of FeTMPyP at the reperfusion stage significantly reduced MMP-9 and MMP-2 expression in ischemic brains[62]. Our recent study showed that $ONOO^-$-mediated MMP-9 activation contributed to hemorrhagic transformation (HT) in a rodent ischemic stroke model with delayed tissue plasminogen activator (t-PA) treatment[84]. Delayed t-PA treatment beyond 4.5 h after MCAO ischemia significantly up-regulated the expression of 3-NT and MMP-9 and aggravated HT in the ischemic brain area. FeTMPyP treatment significantly down-regulated MMP-9 activity, attenuated HT and improved the neurological outcomes[84]. Furthermore, other studies have shown that $ONOO^-$ production mediates the activation of purified human proMMP-1, -8, and -9 in the presence of similar concentrations of GSH via S-nitrosoglutathione[85]. Peroxynitrite activated MMP-2 in the presence of glutathione by modifying the cysteine residue in the auto-inhibitory domain of the zymogen[86]. Taken together, $ONOO^-$-mediated MMP activation plays crucial roles in BBB damage and hemorrhagic transformation during cerebral ischemia-reperfusion injury.

Role of caveolin-1 in acute ischemic brain injury

Caveolae are flask-shaped lipid rafts in the cell membrane, ranging from 50 to 100 nm in size, that regulate transport and cell signaling. Caveolins, which are 19–22-kDa integral membrane proteins located at caveolae, are abundant in adipocytes, endothelial cells, and fibroblasts and are critical for caveolae formation[87-93]. Caveolins have three subtypes including caveolin-1, -2, and -3, with an NH_2-terminal membrane attachment domain (N-MAD, Residues 82–101) and COOH-terminal membrane attachment domain (C-MAD, residues 135–150) that binds to membranes with high affinity[94-96].

Caveolin-1 (Cav-1) binds to all isoforms of NOS via the Cav-binding motif and inhibits NOS activity[97-100]. Caveolin-1 has two cytoplasmic domains including the scaffolding domain (amino acids 61-101) and C-terminal tail (amino acids 135-178), which are able to bind with eNOS. Peptides derived from the scaffolding domains of Cav-1 and Cav-3 inhibited eNOS, iNOS and nNOS activities[101] and subsequently reduced NO production in blood vessels and endothelial cells[99]. Overexpression of Cav-1 significantly attenuated eNOS enzyme activity in endothelial cells[102, 103]. Loss of Cav-1 persistently activated eNOS both in mice and human subjects[104]. Under a transient MCAO ischemia-reperfusion condition, Cav-1 KO mice had a larger infarction volume than wild-type mice[105]. Interestingly, Cav-1 expression was significantly down-regulated in ischemic brains during cerebral ischemia-reperfusion injury compared to that in control brains. NOS inhibitors including *L*-NAME, N6-(1-iminoethyl)-lysine (NIL) and 7-NI all prevented the loss of Cav-1 in ischemic brains[33], indicating that NO down-regulates Cav-1 in ischemic brain injury. The interaction between NO and Cav-1 forms a positive feedback loop for the regulation of NO production in cerebral ischemia-reperfusion injury. Notably, the roles of NO in the modulation of Cav-1 expression appear to be different in neuroblastoma cells. An NO donor up-regulated the expression of Cav-1, while both the non-selective NOS inhibitor *L*-NAME and iNOS inhibitor 1400W abolished the induction of Cav-1 in neuroblastoma SK-N-MC cells. Increased Cav-1 expression may be an adaptive mechanism in neuroblastoma cells in

response to hypoxic stimulation[106]. Consistent results have also been found in lung cancer cells[107]. Thus, the interaction of Cav-1 and NO could be an important cellular signaling pathway to cope with different pathological processes whose defensive or detrimental effects might be related to cell types and pathological conditions.

The interaction of Cav-1 and NO impacts BBB permeability through modulation of MMP activation in cerebral ischemia-reperfusion injury[108]. Cav-1 was co-localized with MMP-2 on the surface of endothelial cells[109, 110]. NO modulated the expression and distribution of Cav-1 and MMP-9 at the endothelial cell/tumor cell interface[111]. Treatment of Cav-1 peptide protected BBB integrity from chemokine-induced damage as evidenced by the up-regulation of TJ and adherent junction proteins in BMECs *in vitro*[112]. Cav-1 KO mice had increased eNOS activity and NO production in endothelial cells along with endothelial hyper-permeability compared to wild-type mice[113]. To explore the roles of Cav-1 in the regulation of BBB permeability, we compared the activities and expression of MMPs and the BBB permeability in a mouse MCAO model. After wild-type mice were subjected to cerebral ischemia-reperfusion injury, Cav-1 expression was down-regulated, accompanied with increased MMP-2 and -9 activities, decreased ZO-1 expression and enhanced BBB permeability in ischemic brains. The roles of Cav-1 in the modulation of MMPs and BBB permeability were further confirmed by using Cav-1 KO mice *in vivo* and Cav-1 RNAi brain microvascular endothelial cells (BMECs) *in vitro*. Knockout or knockdown of Cav-1 aggravated the BBB permeability and cell damage. Furthermore, *L*-NAME treatment partly inhibited MMP activation and protected the BBB integrity in Cav-1 KO mice[114]. The results suggest that NO production directly contributes to MMP activation and BBB disruption even without Cav-1 involvement. Cav-1 only partly contributes to the BBB damage. Similar results have also been reported by others[115, 116]. Lentiviral-mediated re-expression of Cav-1 inhibited MMP activation, protected TJ protein expression and decreased brain edema in Cav-1 KO mice[115]. Thus, we conclude that the NO/Cav-1/MMP signaling cascades play critical roles in mediating BBB damage during cerebral ischemia-reperfusion injury[114]. In addition, peroxynitrite also affected Cav-1 expression in endothelial cells. The expression of 3-NT was co-localized with Cav-1 in the endothelial cells of the diabetes mellitus (DM) patients, and exogenous peroxynitrite decreased the caveolae structure and Cav-1 expression, which led to NOS uncoupling[117]. Interestingly, similar results were also found in hepatic ischemia/reperfusion injury, showing Cav-1 KO mice have more 3-NT expression in liver tissues than wild-type mice[47]. Therefore, the interaction of RNS and Cav-1 may be an important cellular signaling pathway in both cerebral and hepatic ischemia-reperfusion injury[47, 108, 114, 116, 118]. However, controversial results in different neurological disease models have also been reported. In a rat cortical cold-injury model, increased Cav-1 expression and phosphorylation were co-localized with decreased occludin and claudin-5 expression in the brain area with increased BBB permeability[119]. Expression of phosphorylated Cav-1 was increased in endothelial cells after cortical cold injury, which was associated with BBB disruption and edema in brain injury[120]. The exact mechanisms and explanations for those controversial results are unclear. Since those studies only presented a phenomenon in which increased Cav-1 expression co-existed with BBB disruption in the rat cortical cold-injury model, further investigations should be conducted for the proof-of-concept of the roles of Cav-1 in BBB permeability. Recently, we stepped forward to investigate the roles of Cav-1 in the modulation of the BBB permeability in neuroinflammation diseases by using a laboratory murine model of experimental autoimmune encephalomyelitis for mimicking multiple sclerosis. Increased expression of Cav-1 in the serum and spinal cord was associated with disease incidence and severity in wild-type mice with active encephalomyelitis. After immunization, Cav-1 KO mice showed a remarkably lower disease incidence and fewer clinical symptoms than wild-type littermates. The Cav-1 KO mice also had fewer encephalitogenic T cells trafficking into the CNS and decreased expression of adhesion molecules ICAM-1 and VCAM-1 within the lesions. Thus, we concluded that Cav-1 could mediate CNS-directed lymphocyte trafficking across the BBB via interacting with adhesion molecules ICAM-1 and VCAM-1, subsequently aggravating neuroinflammation and degeneration in EAE pathology[121, 122]. The above results indicate that Cav-1 has different functions in different neurological diseases. Particularly for ischemic brain injury, we concluded that the interaction of RNS, Cav-1 and MMPs could form a positive feedback loop, amplifying the impact of RNS in BBB disruption and ischemic brain injury (Figure 1). Therefore, targeting the RNS/Cav-1/MMP pathway is a promising therapeutic strategy for protecting against cerebral ischemia-reperfusion injury[28, 108, 123].

Natural active compounds targeting $ONOO^-$/Cav-1/MMP-9 signaling pathway for neuroprotection in ischemic stroke

Based on abundant experience and accumulated histological evidence, Chinese herbal medicine has been used for the treatment of stroke in China for centuries. Herbal formulas or single herbs are great sources for drug discovery. Herein, we summarize the current progress in the exploration of active compounds from Chinese medicinal herbs that modulate the RNS/Cav-1/MMP signaling pathways and their implications for neuroprotection in the treatment of ischemic stroke.

Calycosin and calycosin-7-*O*-β-*D*-glucoside

Calycosin and its glycoside form calycosin-7-*O*-β-*D*-glucoside (CG) are two representative isoflavones isolated from *Astragali Radix*, a medicinal herb used for ischemic stroke for hundreds of years in China[124]. The chemical structure of CG is shown in Figure 2. We investigated the neuroprotective effects of CG on modulating the NO/Cav-1/MMP signaling pathway and reducing infarction volume and BBB permeability in a rat MCAO cerebral ischemia-reperfusion model[125]. CG inhibited MMP activation, maintained the expression of Cav-1 and tight

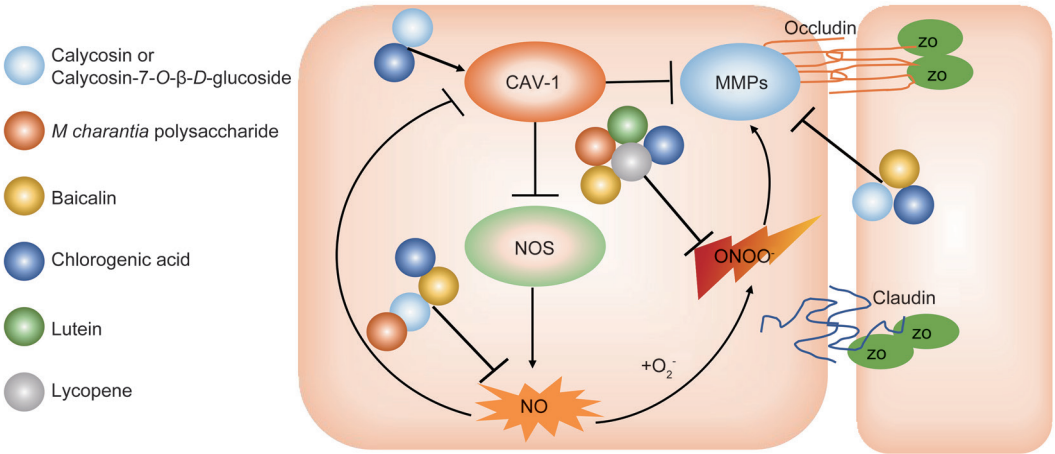

Figure 1. Schematic illustrating the involvement of RNS/caveolin-1/MMPs in mediating the ischemic brain injury and the natural compounds regulating related targets. Upon cerebral ischemia, reduction of caveolin-1 (CAV-1) activates nitric oxide synthase (NOS) and overproduces NO. NO is accumulated and down-regulates CAV-1 expression in ischemic brains, which further activates NOS and forms a feedback interaction to amplify the detrimental signals. MMPs activity is negatively regulated by CAV-1, and loss of CAV-1 leads to higher activity of MMPs. In addition, NO reacts with O_2^- to generate $ONOO^-$. $ONOO^-$ is highly toxic and could also activate MMPs. Active MMPs cleave tight junction proteins, including occludin, claudin, and ZO-1, leading to blood-brain barrier damage. Calycosin or Calycosin-7-*O*-β-*D*-glucoside targets on CAV-1 (Ref 125), NO (Ref 124, 125, 130), and MMPs (Ref 125, 127, 128); *M charantia* polysaccharide targets on NO (Ref 45) and $ONOO^-$ (Ref 45); Baicalin targets on NO (Ref 150, 151), $ONOO^-$ (Ref 46, 59, 151), and MMPs (Ref 46, 152, 153); Chlorogenic acid targets on CAV-1 (Ref 181), NO (Ref 173), $ONOO^-$ (Ref 168–171), and MMPs (172, 175, 176); lutein targets on $ONOO^-$ (Ref 182); lycopene targets on $ONOO^-$ (Ref 187-189). Ref, reference.
⊣ Inhibit
→ Promote

junction proteins, attenuated BBB disruption, reduced infarction volume and improved the neurological outcomes in cerebral ischemia-reperfusion injury[125, 126]. Calycosin also demonstrated bioactivities of scavenging free radicals and inhibiting MMP-9 activity in other cellular or non-cellular systems[127-129]. Calycosin and calycosin-7-*O*-β-*D*-glucoside decreased the production of NO, O_2^-, and TNF-α in lipopolysaccharide (LPS)-stimulated microglial or RAW 264.7 macrophages[124, 130] and attenuated the neurotoxicity induced by various pathological factors including LPS, glutamate, mongholicus and xanthine (XA)/xanthine oxidase (XO)[130-132]. In addition, calycosin attenuated the permeability of human umbilical vein endothelial cells (HUVECs) under hypoxic conditions, possibly through inhibiting ROS production and preserving cytoskeleton structure[133]. These results suggest that the inhibition of the RNS/MMP-9 signaling pathway contributes to the neuroprotective and vascular protective effects of calycosin and CG. Nevertheless, other mechanisms could also account for the neuroprotective effects of calycosin and CG. For example, calycosin up-regulated transient receptor potential canonical 6 (TRPC6) and induced phosphorylation of CREB in ischemic brains[134]. Calycosin modulated the positive feedback of estrogen receptor ER-α and microRNA-375 in cerebral ischemia-reperfusion injury[135]. Calycosin was shown to act as a non-competitive calcium channel blocker to prevent calcium overload[136]. CG activated the PI3K/Akt pathway and had neuroprotective effects in cerebral ischemia-reperfusion injury[137]. Therefore, calycosin and CG are able to modulate multiple signaling targets to exert their neuroprotective effects on ischemic brain injury. With better bioavailability than calycosin, CG has greater potential for further translational research[125].

Baicalin

Baicalin is one of the major flavonoids isolated from the dried root of *Scutellaria baicalensis*, a medicinal herb used for ischemic stroke in China[138]. The chemical structure of baicalin is shown in Figure 3. Baicalin promoted neuronal differentiation of neural progenitor cells[139, 140]. Baicalin reduced brain infarction volume, brain edema, BBB damage and brain inflam-

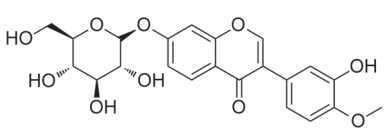

Figure 2. Calycosin-7-*O*-β-*D*-glucoside.

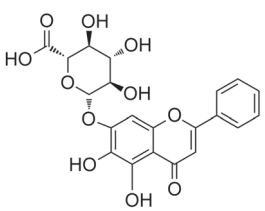

Figure 3. Baicalin.

mation in rodent ischemic stroke models[138, 141-145]. Baicalin decreased BBB permeability and protected brain microvascular endothelial cells (BMVECs) *in vivo* and *in vitro*[46, 145, 146] and reduced neurotoxicity under an OGD condition[147-149]. The neurovascular protective effects of baicalin may be attributed to its antioxidant effects[138, 150, 151]. By using mass spectrometry and EPR spin trapping experiments, we demonstrated the direct scavenging activities of baicalin on $ONOO^-$ and superoxide[59]. Baicalin inhibited the formation of 3-nitrotyrosine in ischemic brain tissues[59]. By using our newly developed peroxynitrite-specific probe, HK-Yellow AM, we directly visualized the production of $ONOO^-$ in ischemic brains. Baicalin also inhibited $ONOO^-$ production and reduced MMP-9 activity[46], protected the expression of the tight junction protein occludin, and attenuated the BBB damage and brain edema in both permanent MCAO model and intracerebral hemorrhage model[152, 153]. Notably, a proteomic study and gene microarray indicated that baicalin acted in a regulatory network to induce its neuroprotective effects against cerebral ischemic injury[142, 154]. Further studies revealed that baicalin inhibited toll-like receptor 2/4 and NF-kB pathways[143, 151, 155], reduced the phosphorylation of CaMKII[156] and up-regulated AMPK alpha signaling[157]. Thus, baicalin is a good drug candidate for the treatment of stroke[142, 158, 159].

M charantia polysaccharide (MCP)

M charantia polysaccharide (MCP) is one of the important bioactive components of *Momordica charantia* (MC), also named the bitter melon. MC has antioxidant and anti-hyperglycemic effects on cerebral ischemia-reperfusion injury in diabetic mice[160]. MCP showed its antioxidant effects through promoting endogenous antioxidant enzyme activities in a rat myocardial infarction model[161, 162]. Our recent studies showed that MCP dose-dependently reduced infarction volume and attenuated neuronal apoptosis in animal models of four-vessel occlusion (4-VO) and MCAO. MCP had scavenging effects on NO and $ONOO^-$, inhibited the release of cytochrome *c* from mitochondria and modulated the activation of the JNK3, c-Jun, and Fas-L signaling pathways in ischemic brains[45]. NO was reported to mediate the activation of JNK3 signaling via S-nitrosylation, and antioxidant N-acetylcysteine down-regulated JNK3 signaling and protected neurons from ischemic brain injury[163, 164]. Thus, the neuroprotective effects of MCP may be attributed to inhibiting the free radical-mediated c-Jun N-terminal kinase 3 signaling pathway to protect against cerebral ischemia-reperfusion injury.

Chlorogenic acid

Chlorogenic acid (CGA) is a dietary phenylpropanoid molecule derived from a variety of natural products such as aubergine, blueberries and coffee[165-167]. The chemical structure of CGA is shown in Figure 4. CGA directly reacted with $ONOO^-$ with a rate constant of $1.6\pm0.7\times10^5\ M^{-1}\cdot s^{-1}$ and prevented DNA damage[168-171]. CGA is also a strong MMP-9 inhibitor with an IC_{50} of 30–50 nmol/L[172]. CGA inhibited the excess production of NO in LPS/gamma-interferon (IFN-gamma)-treated C6 astrocytes[173]. CGA improved the behavioral outcome in a rabbit small clot embolic stroke model[174]. CGA and its metabolite dihydrocaffeic acid (DHCA) inhibited MMP-2/9 activity, attenuated BBB damage and reduced brain infarction and brain edema[175, 176]. CGA also exerted anti-inflammatory effects against cerebral ischemia-reperfusion injury[177, 178]. CGA protected against glutamate-induced neurotoxicity in primary cultured cortical neurons[179]. CGA was shown to cross the BBB[180]. In an alcoholic liver injury model, CGA up-regulated the expression of Cav-1 and inhibited Stat3/iNOS signaling and hepatic lipid accumulation and peroxidation[181]. Thus, CGA could target the RNS/Cav-1/MMP signaling pathways to potentially protect the brain against ischemic stroke. Notably, CGA has synergistic effects with tissue plasminogen activator (t-PA), the only FDA-approved drug, in improving the neurological outcomes[174]. As a thrombolytic treatment, t-PA has a restrictive therapeutic time window within 4.5 h, and treatment beyond this time window increases the risk of hemorrhagic transformation (HT)[67]. BBB disruption is a critical process of delayed t-PA-induced HT, which involves $ONOO^-$ generation and MMP activation[67, 84]. With the bioactivities of inhibiting $ONOO^-$ and MMPs, further study of the potential of CGA as an adjunct agent for protecting BBB integrity and preventing t-PA-mediated HT during thrombolytic treatment for ischemic stroke is valuable.

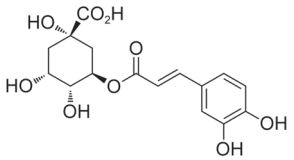

Figure 4. Chlorogenic acid.

Other compounds

Other compounds such as lutein and lycopene may also target RNS to protect ischemic brains. For example, lutein (Figure 5), a xanthophyll rich in green leafy vegetables, directly reacted with peroxynitrite and nitrogen dioxide radicals[182] and protected human neuroblastoma cells from DNA damage induced by peroxynitrite[183]. Lutein treatment ameliorated oxidative stress and inflammation, reduced brain infarction volume and protected against neuronal apoptosis in mouse MCAO models[184, 185]. Interestingly, ischemic stroke patients with a poor early outcome showed significantly lower plasma lutein levels than those who remained functionally stable[186]. These results suggest that lutein may protect ischemic brains through its antioxidant effects. As lutein is a safe daily supplement for ocular health, its potential application for stroke treatment merits further study.

Lycopene (Figure 6) is another compound that has been shown to directly react with $ONOO^-$. Lycopene prevented protein nitration and DNA damage in lung fibroblast cells[187-189]. Lycopene exerted antioxidative stress effects and inhibited neuronal apoptosis in rodent transient cerebral isch-

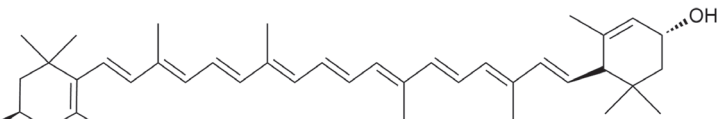

Figure 5. Lutein.

emia/reperfusion models[190, 191]. A recent meta-analysis of 116 127 participants and 1989 cases showed that circulating lycopene was negatively associated with the risk of stroke[192]. Similarly, a prospective study also demonstrated the potential of lycopene for reducing the risk of stroke onset[193]. Hence, lycopene is valuable for further studies as a neuroprotective agent for ischemic stroke. In addition, other compounds, such as resveratrol, curcumin, apocynin, caffeic acid, and tanshinone IIA, have been noted as good candidates for inhibiting RNS-mediated brain damage in cerebral ischemia-reperfusion injury or ischemic brain injury. We have reviewed their values as potential therapeutic agents in our previous articles[28, 82, 158]. The details have been discussed before and should not be repeated here.

Discussion

Ischemic stroke is a major cause of death and long-lasting disability worldwide. Identifying new therapeutic targets is important for drug development to treat ischemic stroke. In this review, we have highlighted the important role of the RNS/Cav-1/MMP pathway in mediating cerebral ischemia-reperfusion injury (Figure 1). As shown in Figure 1, we have summarized the effects of several representative compounds targeting the RNS/Cav-1/MMP signaling pathway to demonstrate their neuroprotective mechanisms in ischemic stroke treatment.

The following points regarding studies on drug discovery for ischemic stroke should be noted. First, optimal therapeutic strategies should be considered since RNS has complex functions at different stages of stroke pathology. For example, at a low concentration, free radicals could also contribute to redox signaling. NO can be a cellular signaling molecule that promotes neuronal proliferation and migration and improves neurological outcomes at the recovery stage in post-ischemic brains[194]. MMP-9 has also been shown to exert its beneficial effects on neuronal plasticity and brain remodeling at the recovery phase in post-stroke brains[195]. Treatment with an MMP-9 inhibitor beginning at day 7 exacerbated brain injury and impaired the functional outcomes of rats at day 14 after MCAO ischemia[195]. Consistently, we found that caveolin-1 inhibited the neuronal differentiation of neural stem cells via the VEGF pathway, and Cav-1 KO mice showed more abundant newborn neurons in brains[196]. Cav-1 KO mice revealed a better proliferation capacity of adult neural stem cells than wild-type mice[197]. Hence, the RNS/Cav-1/MMP pathways may be beneficial for brain repair at the recovery phase of ischemic stroke. For drug treatment, the half-life of the aforementioned compounds is quite short, within several hours, which is still in the acute phase of stroke. For example, calycosin-7-*O*-β-*D*-glucoside had an elimination half-life of approximately 2.18 h after oral gavage in rats[198]. Baicalin had an elimination half-life of 0.12 h after intravenous injection in rats[199]. The CGA metabolite level was decreased to less than 50% within 1.5 h after intraperitoneal injection[200]. To reach the goal of the best neuroprotective outcome without interrupting the brain repair process, the selection of the optimal intervention time, dosage and frequency by integrating the knowledge of the functions of cellular signaling pathways at different stages of brain damage and repair and the pharmacological activities, pharmacokinetics and pharmacodynamics of those compounds is important[69, 201]. Thus, understanding the dynamic changes of RNS, Cav-1 and MMPs and their impact on brain injury and brain repair after ischemic stroke is a prerequisite.

We should also consider the therapeutic time window of these compounds. In current studies, most of the compounds were applied within 2 h after ischemia onset[59, 125]. In the past, many neuroprotective compounds have failed in clinical trials despite animal studies showing promising neuroprotective effects. One of the important reasons for this failure in clinical trials might be the limited therapeutic time windows of those compounds[202]. A compound that shows a neuroprotective effect when treated at 2 h after experimental stroke attack may not guarantee its therapeutic effect on stroke patients, especially when those treatments are launched several hours after stroke onset. Therefore, the compounds that show a broad therapeutic time window in an experimental stroke model are favorable. A series of experiments should be conducted to determine the therapeutic time window of those compounds in stroke treatment.

For drug development, we should consider the following key issues. First, the pharmacokinetics and pharmacodynamics of the candidate compounds should be taken into consideration. The BBB is a critical factor limiting drug distribution into the brain[203, 204]. The BBB penetration of the drug is usu-

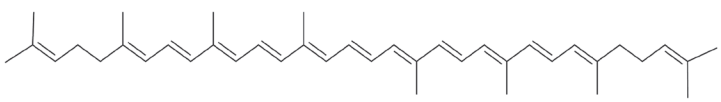

Figure 6. Lycopene.

ally determined by two major parameters: one is the physicochemical properties of the compounds (such as molecular weight, rotatable bonds, solvent-accessible surface areas, H-bond capacity), and the other is the binding affinity of the compounds to the central nervous system (CNS) drug efflux pumps (most often the P-glycoprotein)[204]. Some simple rules based on the physiochemical features help to predict the BBB penetration of the compounds. For example, if the number of N+O in a compound is no more than five, the compound is likely to cross the BBB[205]. A CNS drug has been proposed to have an *in vitro* passive permeability more than 150 nm/s and should not be a good P-glycoprotein substrate[206]. Nevertheless, experimental data should be collected to directly show the penetration of the compounds into the CNS. Cerebral-spinal fluid (CSF) studies are usually conducted to calculate the CNS exposure of drug candidates[204]. An animal study showed that baicalin could cross the BBB and was detected in the CSF after single intravenous injection at a dosage of 24 mg/kg[207]. CGA was also detected in the CSF of rats after oral administration and reached the level of pharmacological effect[208]. These preclinical results suggest the potential of these compounds to enter the ischemic brains to exert their neuroprotective effects.

Another key issue is the acquisition of direct evidence of the compound-target interaction in the treatment of ischemic brain injury[204]. Although many papers have reported the therapeutic effects of candidate compounds with *in vivo* and *in vitro* data, most of the aforementioned studies did not provide data regarding the direct interaction of the active compounds with the observed targets, such as the RNS/Cav-1/MMP signaling pathway, in the experimental systems. Moreover, we should note that those compounds might have multiple targets, and their neuroprotection should not be simply explained by targeting a single signaling pathway. For example, calycosin-7-*O*-β-*D*-glucoside also modulated TRPC6 and ER-α signaling in ischemic brain injury[134, 135]. Baicalin was also revealed to inhibit TLR-2/-4 signaling and attenuate brain inflammation[143]. Therefore, further exploration of the molecular targets of natural compounds and differentiation of the direct and indirect effects of those compounds on certain cellular signaling pathways and disease progression are desirable.

Recent advances in brain imaging technology highlight positron emission tomography as a useful tool to directly assess drug distribution and drug-target interaction[204, 209]. The technique enables scientists to further evaluate the binding affinity and efficacy of the compounds to a target of interest in different brain regions among different species[209, 210]. By using this method, we could guide the dosage selection by determining the target occupancy and its relationship to the blood-drug concentration[210].

The third key issue is the downstream biological effects of these compounds in human subjects. Preclinical studies of those compounds seem promising. To evaluate the overall drug efficacy, clinical trials are needed to evaluate neurological scores, brain infarction, brain edema, etc. In addition, measurements of serum biomarkers will help assess the modulation of related pathways as well as the drug effectiveness. For example, 3-NT and MMP-9 are potential biomarkers of ischemic stroke and are associated with the prognosis of stroke outcomes[66, 73]. Therefore, serum 3-NT and MMP-9 levels may help monitor the effects of the compounds on the RNS/caveolin-1/MMP-9 signaling pathways.

In summary, we propose that the RNS/Cav-1/MMP pathway plays an important role in mediating cerebral ischemia-reperfusion injury. Targeting this novel signaling pathway could provide a new clue for drug discovery for the treatment of ischemic stroke.

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