Review

Targeting ONOO[−]/HMGB1/MMP-9 Signaling Cascades: Potential for Drug Development from Chinese Medicine to Attenuate Ischemic Brain Injury and Hemorrhagic Transformation Induced by Thrombolytic Treatment

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Key Words

Chinese medicine · Hemorrhagic transformation · Ischemic stroke · Tissue plasminogen activator · Natural compounds · Peroxynitrite · High-mobility group box 1 protein · Matrix metalloproteinase-9

Abstract

Stroke is the leading cause of death and disability worldwide, and ischemic stroke accounts for more than 85% of the stroke incidence. Tissue plasminogen activator (t-PA) is the only FDA-approved drug for ischemic stroke treatment with a narrow treatment time window of 4.5 h. Hemorrhagic transformation (HT) is a severe complication of delayed t-PA treatment in ischemic stroke. Thus, it is critically important to develop combination therapies to reduce HT and extend the therapeutic time window of t-PA. Current progress suggests that peroxynitrite (ONOO[−])/high-mobility group box 1 protein (HMGB1)/matrix metalloproteinase-9 (MMP-9) signaling cascades could be important for attenuating HT during thrombolytic treatment for acute ischemic stroke. Recently, important progress has been made in seeking for natural compounds from Chinese medicine for reducing ischemic stroke injury, with some of them targeting ONOO[−]/HMGB1/MMP-9 signaling cascades. Herein, we analyze the roles and interactions of these three targets in mediating HT; subsequently, we summarize the potential compounds from Chinese herbal medicine for attenuating HT and analyze the related targets. Finally, we raise the potential issues to be addressed in further development of these compounds as combination therapy.

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Introduction

Stroke is the second leading cause of death, and ischemic stroke accounts for about 85% of stroke cases [1]. Tissue plasminogen activator (t-PA) remains the only FDA-approved therapy for acute ischemic stroke [2]. Early recanalization with t-PA infusion indeed improves the outcome of ischemic stroke, as suggested by observational studies [3], placebo-controlled trials [4, 5] and meta-analyses [6, 7]. However, t-PA has a restrictive time window of 4.5 h [8]. Beyond this, t-PA treatment increases 10-fold the risk of hemorrhagic transformation (HT), a bleeding into the ischemic brain, which accounts for 10–40% of ischemic stroke cases with increased morbidity and mortality [9]. Therefore, developing methods to reduce HT is critically important to extend t-PA's therapeutic window and increase its eligibility for ischemic stroke.

Recent studies propose potential solutions by using combination therapies with t-PA to reduce HT and improve therapeutic outcomes [10, 11]. Those therapies mainly target matrix metalloproteinase-9 (MMP-9), which is an important target mediating HT [11]. In our previous study, we have reported that reactive nitrogen species (RNS), especially peroxynitrite (ONOO[−]), play an important role in mediating MMP-9 activation and HT in a rat middle cerebral artery occlusion (MCAO) model [12]. In the process, high-mobility group box 1 protein (HMGB1) appears to be a critical signaling molecule to interact with ONOO[−] and MMP-9 [13, 14]. The interaction of HMGB1, ONOO[−] and MMP-9 participates in the pathological process in cerebral ischemia injury. Hence, strategies targeting this signaling cascade could be promising for reducing t-PA-induced HT in ischemic stroke.

Research on adjunct therapy has not been translated into clinical practice yet. Choosing low-toxicity agents is important for the development of adjunct therapy and its further translation into clinical practice. Chinese medicine has been used for treatment of ischemic stroke for thousands of years. Interestingly, many active compounds from Chinese medicine have been demonstrated with good safety profiles, some of which are FDA approved, such as baicalin and catechin [15]. More importantly, studies on herbal compounds revealed their strong protective effects by reducing cerebral ischemia-reperfusion injury, including decreasing infarct volume, brain edema and blood-brain barrier (BBB) damage, suppressing brain inflammation, and improving the neurological outcomes [16, 17]. Some of the compounds, such as baicalin, tanshinone IIA and resveratrol, could inhibit ONOO[−]/MMP-9/HMGB1 signaling cascades in ischemic stroke models, which indicates their potential for reducing HT.

Herein, we first summarize the roles of ONOO[−], HMGB1 and MMP-9 in mediating HT with delayed t-PA treatment in ischemic stroke. Based on these targets, we discuss the potential of representative single compounds from Chinese medicine as combined therapy with t-PA to reduce HT, with a focus on the modulating effects of those compounds on ONOO[−], HMGB1 and MMP-9. Finally, we discuss the important issues that need to be addressed in future development of those compounds as adjunct therapies.

Mechanisms of t-PA-Induced HT

Multiple mechanisms have been identified which mediate HT after delayed t-PA treatment in ischemic stroke, as addressed by several review articles [10, 18–21]. Among various targets, MMP-9 is a well-studied and established factor contributing to HT in both human and animal studies [22–24]. Recently, we found that ONOO[−] also significantly participated in mediating HT, possibly via regulating MMP-9 activation in an ischemic stroke model with t-PA treatment [12]. In addition, HMGB1, which is an important molecule in mediating brain inflammation, was also shown to induce MMP-9 production and BBB damage [13]. ONOO[−],

HMGB1 and MMP-9 may synergistically contribute to HT during ischemic stroke with t-PA treatment. In the following section, we focus on these three targets and discuss their potential interaction as a pathway in inducing HT.

Role of ONOO[−] in HT

Recanalization after t-PA infusion causes cerebral ischemia-reperfusion injury. Ischemic brains produce a large amount of reactive oxygen species (ROS) and reactive nitrogen species (RNS) [25]. Superoxide (O₂[−]) and nitric oxide (NO) are representative free radicals in cerebral ischemia-reperfusion injury. They can react with each other extremely rapidly to form ONOO[−] with a diffusion limit ($k_2 = 4.7 \times 10^9 \text{ m}^{-1}\text{s}^{-1}$). ONOO[−] was increased at about 4 h and peaked at around 48 h after reperfusion [26]. ONOO[−] induces tyrosine nitration of proteins through adding the nitro group to the hydroxyl group of tyrosine residues [27]. The nitrotyrosine level in the peri-infarct area was about two times higher than that in the core area at 1 h of reperfusion after ischemia, and persisted until 48 h after reperfusion [28]. Consistently, 3-nitrotyrosine was also increased in the plasma of stroke patients [29]. ONOO[−] has far more activity than its precursors [30, 31]. For example, ONOO[−] easily penetrates the lipid layers of the cell membrane, inhibits mitochondrial respiration [32, 33], induces protein nitration [34, 35], lipid peroxidation [36], causes DNA damage [37] and inactivates multiple ion channels and enzymes [38, 39]. Peroxynitrite decomposition catalysts (PDCs) can neutralize ONOO[−] to form nontoxic nitrates. FeTMPyP, a representative PDC, significantly prevented neuronal apoptosis and death, reduced the cerebral infarct size and improved functional outcomes in rodent transient ischemic stroke models, with a therapeutic time window up to 6 h [40, 41]. Recently, we found that FeTMPyP co-treatment with t-PA significantly reduced HT induced by delayed t-PA treatment in a rat MCAO model [12]. The protective effect of FeTMPyP could be attributed to inhibiting ONOO[−]-mediated MMP-9 production and activation [12]. Uric acid is an ONOO[−] scavenger, considered as a potential neuroprotective agent for ischemic stroke treatment [42]. Uric acid treatment significantly reduced the infarct size by >70% in a rat MCAO model [43]. A combination of uric acid with t-PA synergistically reduced the cerebral infarct volume and attenuated neurological deficits in a rat thromboembolic model [44]. Moreover, a recent meta-analysis including 10 studies with 8,131 ischemic stroke patients revealed that high serum uric acid was associated with good neurological outcomes [45]. These works strongly indicate that ONOO[−] plays an important role in mediating t-PA-associated HT and neurological deficit.

Role of MMP-9 in HT

BBB damage is a critical process of HT, and MMP-9 activation is important for BBB opening [23]. Pro-MMP-9 is a 92-kDa type IV collagenase, and the propeptide domain is removed when pro-MMP-9 is activated [46]. Active MMP-9 is capable of degrading the neurovascular matrix, including collagen, fibronectin and laminin, and disrupting tight junctions [47–50]. MMP activation and BBB opening simultaneously occur in ischemic brains. After stroke, plasma MMP-9/-2 activities were increased within 3–8 h, and early BBB opening occurred within 3–6 h, following early HT at 18–24 h [51, 52]. Neutrophils are a critical source of MMPs in BBB disruption in ischemic brain injury [51]. Within 30 min of cerebral ischemia, circulating leukocytes adhere to vascular endothelial cells; by 6 h, neutrophils have entered ischemic brains [23]. Increased MMP-9 mRNA was found in human peripheral leukocytes at 3–5 h after stroke, and the peak of MMP-9 activity was at 6–8 h and returned to baseline by 24 h [23]. MMP-9 activity was also significantly enhanced in human ischemic brains [53, 54]. Moreover, MMP-9 in the ischemic core was found to be mostly localized to the blood vessels, infiltrated neutrophils and microglia, while MMP-9 in the peri-infarct area was greatly localized to microglial cells [54].

MMP-9 plays an important role in cerebral ischemia injury. Broad-spectrum or specific MMP-9 inhibitors significantly reduced neuronal damage in cerebral ischemia models [55, 56]. MMP-9-neutralizing antibody also significantly reduced the brain infarct volume after cerebral ischemia [57]. Consistently, MMP-9 knockout mice showed attenuated brain infarct after cerebral ischemia [58]. More importantly, MMP-9 greatly contributes to BBB damage during cerebral ischemia [24, 59]. MMP-9 knockout in mice protected from BBB disruption in a cerebral ischemia model [58]. MMP-9 siRNA or shRNA treatment reduced Evans blue and IgG extravasation at 24 h of reperfusion in rodent ischemic stroke models [60, 61]. In addition, broad-spectrum or specific MMP-9 inhibitors also reduced BBB damage in cerebral ischemia models [49, 56, 62]. Those lines of evidence indicate that MMP-9 plays an important role in mediating BBB damage during ischemic stroke.

It has been reported that treatment with t-PA promoted neutrophil degranulation and MMP-9 release [63], and significantly upregulated MMP-9 levels in ischemic brains [64]. t-PA knockout mice showed lower MMP-9 levels in ischemic brains than wild-type mice [65]. MMPs are responsible for degradation of the extracellular matrix around cerebral blood vessels and neurons, leading to BBB opening and hemorrhage [52]. Inhibition of MMPs with broad-spectrum inhibitors significantly decreased the incidence of delayed t-PA treatment-induced HT and mortality in rodent ischemic stroke models [64, 66–69]. Those preclinical studies suggest that MMP-9 mediates delayed t-PA-induced HT in ischemic stroke. Clinical trials indicate that early plasma MMP-9 level is correlated with infarct severity and BBB damage in stroke patients, and the MMP-9 level at 3 h could predict hemorrhagic complications [70, 71]. Therefore, MMP-9 is an important molecular target in mediating HT in ischemic brains with delayed t-PA treatment.

Role of HMGB1 in HT

Recent progress draws attention to the roles of HMGB1 in leukocyte adhesion, MMP activation and BBB disruption in ischemic stroke [23]. HMGB1 is a single-polypeptide chain with 215 amino acids containing two positively charged DNA binding motifs, HMG-box A (residues 1–79) and B (residues 89–163), and an acidic C terminus (residues 186–215) with aspartic or glutamic acid residues [72, 73]. HMGB1 serves not only as an architectural transcription factor in the nucleus but also as extracellular signaling after release [74, 75]. HMGB1 can either be secreted from activated macrophages, dendritic cells and natural killer cells or passively leaked from necrotic or injured cells [76, 77]. Active release of HMGB1 involves posttranslational modification of HMGB1, including hyperacetylation of lysine residues [77, 78], phosphorylation of serine residues [79] and oxidation of cysteine residues [80]. After release, HMGB1 can bind with RAGE and Toll-like receptors (TLR2, TLR4) and trigger inflammation by inducing cytokines [81]. Elevated HMGB1 was found early at 2–4 h in ischemia-reperfused brains [75, 82]. HMGB1 promoted neuroinflammation in rats with intracerebral hemorrhage (ICH) [83]. Antibody against HMGB1 reduced BBB permeability and infarct volume in rodent cerebral ischemic models [82]. As an HMGB1 inhibitor, glycyrrhizin binds to HMGB1 and inhibits its cytokine-promoting activities. Glycyrrhizin attenuated oxidative stress-mediated MMP activity, inhibited inflammation and apoptosis in ischemic brains [84], and reduced ICH-induced cellular injury [85]. However, a recent study reported that HMGB1 promoted fibrinolysis and reduced t-PA-mediated neurotoxicity [86]. The controversial effects of HMGB1 might be related to the amount of HMGB1 release from cells and its micro-environment.

Relationship among ONOO[−], HMGB1 and MMP-9

As mentioned, ONOO[−], MMP-9 and HMGB1 are important mediators of cerebral ischemia injury and BBB disruption. They intimately interact with each other, which may work together

as a pathway in mediating HT induced by delayed t-PA treatment in ischemic stroke. In the following section, we will discuss the interaction between these three factors.

ONOO[−] Regulates MMP-9

There is an intrinsic relationship between ONOO[−] and MMP-9. ONOO[−]-activated MMPs lead to tight junction disruption and neurovascular unit damage during ischemic stroke [87–89]. ONOO[−] may induce MMP-9 expression by activating NF-κB [90]. ONOO[−] induced pro-MMP-9 activation in the presence of glutathione via S-glutathionylation [91]. FeTMPyP, a representative PDC, decreased MMP-9 activity and protected from neurovascular injury in ischemic stroke [92]. Consistently, FeTPPS, another PDC, suppressed MMP-9 activation and improved the neurological outcome in hemoglobin-induced hemorrhagic stroke models [93, 94]. In addition, uric acid reduced the serum active MMP-9 level of ischemic stroke patients, indicating that ONOO[−] could be associated with MMP-9 in human ischemic stroke [95]. These works suggest that ONOO[−] activates MMP-9 in ischemic stroke, contributing to neurovascular unit damage and HT.

ONOO[−] Regulates HMGB1

HMGB1 activation highly depends on its redox state [96]. The NADPH oxidase-mediated HMGB1 signaling pathway contributes to cerebral ischemia-reperfusion injury. Edaravone, an antioxidant, exerted neuroprotective effects through inhibiting HMGB1 signaling [97]. Baicalin, a flavonoid isolated from the medicinal plant *Scutellaria baicalensis* Georgi, was reported to inhibit HMGB1 release and improve survival in experimental sepsis [98]. In infarct myocardium, PDCs suppressed HMGB1 and decreased infarct size, suggesting that ONOO[−] could induce HMGB1 release from cardiac cells [99]. As an ONOO[−] scavenger, uric acid prevented translocation of HMGB1 release from endothelial cells [100]. However, the relationship between ONOO[−] and HMGB1 release in ischemic brains is still unclear, and the impact of HMGB1 release on BBB disruption and HT in delayed t-PA treatment remains unknown and needs further investigation.

HMGB1 Regulates MMP-9

It is reported that HMGB1 could promote MMP-9 expression from astrocytes and neurons in ischemic brains, predominantly via TLR4 [13]. HMGB1 is closely related to oxidative stress, MMP activation and BBB disruption [13, 83, 84, 96]. In ischemic stroke patients, an increased plasma HMGB1 level was correlated with MMP-9 level and poor clinical outcome [101].

According to those studies, ONOO[−] may induce HMGB1 activation, subsequently mediating MMP-9 activity and expression and inducing BBB damage and HT. Though the interaction of those three factors needs further study, it is clear that they all significantly contribute to cerebral ischemia injury and BBB disruption, eventually leading to HT. Thus, therapies acting on those targets are promising in reducing HT and extending the therapeutic time window of t-PA.

Active Compounds from Chinese Herbal Medicine for Targeting ONOO[−]/HMGB1/MMP-9 Signaling Cascades and Potential for Treating Acute Ischemic Stroke

Traditional Chinese medicine (TCM) has been practiced for more than 2,000 years, and more than 100 herbs are used for treating ischemic stroke [102]. A recent summary of systematic review articles suggests that TCM has beneficial effects on stroke though high-quality clinical trials are needed [103]. Natural products from Chinese herbal medicine have been considered as important sources of drug discovery [104]. Many natural compounds

Fig. 1. The structure of baicalin.

revealed their bioactivities in ameliorating cerebral ischemic injury [105]. Hence, the development of natural compounds from Chinese medicine is a promising direction for protecting ischemic brains. Herein, we present a representative selection of compounds based on the targets discussed, including ONOO[−], HMGB1 and MMP-9. Totally, 7 compounds are selected, and their potential for reducing HT, BBB damage and brain injury are discussed in the following section.

Baicalin

Baicalin is a flavonoid compound isolated from Chinese medicine radix of *S. baicalensis* Georgi, a commonly used TCM herb for removing interior heat according to TCM theory [106]. *S. baicalensis* Georgi is usually used for attenuating various kinds of bleeding due to the accumulation of interior heat in TCM pathology. Baicalin is a flavone, and its chemical structure is shown in figure 1. A recent systemic genome microarray analysis revealed that baicalin treatment regulated multiple pathways in an MCAO model [107]. Our group and others showed that baicalin treatment decreased the cerebral infarct volume, attenuated neurotoxicity and suppressed neuroinflammation both in transient and permanent rodent cerebral ischemia models [108–110]. Baicalin exerts anti-inflammatory effects via modulating the TLR4/NF-κB pathway and downregulating its downstream factors, including inducible nitric oxide synthase (iNOS), cyclooxygenase-2, IL-1β and TNF-α in permanent and transient cerebral ischemia models [110–112]. Antiapoptotic effects of baicalin were also observed in a focal cerebral ischemia injury model, possibly via downregulating protease-activated receptor-1 [113]. In addition to ischemic stroke models, baicalin also exerted protective effects on hemorrhagic stroke models and reduced brain edema and apoptosis in a time- and dose-dependent manner [114].

Previously, we found that baicalin could directly scavenge ONOO[−], as evident by mass spectrometry analysis. Moreover, baicalin reduced ONOO[−] in human neuroblastoma SH-SY5Y cells and rat ischemic brains, subsequently attenuated neurotoxicity and reduced the brain infarct volume [108]. In addition, baicalin also reduced NO and O₂[−] in tert-butyl hydroperoxide-treated endothelial cells, and hence prevented the formation of ONOO[−] [115]. Baicalin could inhibit iNOS and cyclooxygenase-2, which may account for reduced NO and O₂[−] [115]. In addition to scavenging ROS and RNS, baicalin treatment at 2 and 12 h after MCAO onset reduced MMP-9 expression, protected tight junction proteins, attenuated BBB damage and decreased brain edema in a rat MCAO model [116]. Consistently, baicalin also inhibited MMP-9 expression in rat ICH brains, reducing BBB damage and brain edema [117]. The exact mechanisms of how baicalin inhibits MMP-9 expression are still not clear, and several putative targets may be involved. Inhibition of NF-κB may reduce the transcription of MMP-9 gene in stroke models, eventually reducing MMP-9 expression [117]. Inhibition of the p38 MAPK pathway may also account for the suppression of MMP-9 expression [118]. Recently, we

Fig. 2. The structure of resveratrol.

found that inhibiting ONOO[−] with PDC significantly reduced MMP-9 expression [12]. Therefore, scavenging ONOO[−] and suppressing MMP-9 could be the critical molecular mechanisms contributing to the neuroprotective effects of baicalin in cerebral ischemia reperfusion injury.

Recent studies indicate that baicalin could inhibit HMGB1 release in sepsis and vascular disease models [98, 119]. Baicalin also inhibited the release of HMGB1 in lipopolysaccharide (LPS)-challenged human endothelial cells, subsequently reducing the production of the proinflammatory cytokines TNF- α and IL-6, and inhibiting vascular hyperpermeability and leukocyte migration [119]. Similarly, baicalin inhibited HMGB1 release in LPS-treated macrophages [98]. Baicalin did not affect the transcription or translation of HMGB1; rather, it inhibited the cytoplasmic translocation of HMGB1 in macrophages [98]. It seems that several mechanisms may be involved in inhibiting HMGB1 translocation. Baicalin was shown to enhance histone deacetylase (HDAC) expression and activity in chronic obstructive pulmonary disease [120]. HDACs are important molecules for suppressing HMGB1 release [121, 122]. Hence, increasing HDACs may be one of the possible mechanisms of baicalin in inhibiting HMGB1 translocation. Protein kinase C (PKC) activation could lead to phosphorylation of HMGB1 and subsequent translocation [79]. Baicalin inhibited PKC in brain microvascular endothelial cells and hippocampal slices in vitro under oxygen glucose deprivation condition or with NMDA treatment [123, 124]. Suppression of free radical-mediated oxidative stress could also inhibit HMGB1 release as suggested by previous research [97]. However, whether baicalin can inhibit HMGB1 release in a stroke model needs further investigation.

As baicalin reveals pleiotropic effects by attenuating cerebral ischemic injury and has the capacity to target ONOO[−]/HMGB1/MMP-9 signaling cascades, we propose that this compound is a promising candidate for combination therapy with t-PA in treating acute ischemic stroke.

Resveratrol

Resveratrol is a natural phenol found in the Chinese herb *Polygonum cuspidatum* and other sources such as grapes and eucalyptus [125]. The structure of resveratrol is shown in figure 2. Resveratrol is a potential drug candidate for treatment of ischemic stroke, as summarized in a recent review article [126]. Resveratrol exhibited multiprotective effects, such as antiapoptotic [127, 128], antioxidative [129], anti-inflammatory [130] and antiexcitotoxic effects [131, 132]. Resveratrol has a therapeutic time window up to 6 h after ischemic onset; it reduced infarct volume, attenuated microglial activation and suppressed cytokine production and free radical generation [130]. Proteomic analysis revealed that resveratrol treatment in a rat MCAO model regulated various proteins related to oxidative stress and energy metabolism [133]. Pretreatment with resveratrol even 14 days before MCAO reduced the brain infarct volume by 33% in rats, which was mediated by silent mating type information regulation 2 homolog 1 (SIRT1) [134]. Nrf2 activation is another mechanism of resveratrol which mediates preconditioning protective effects in a rodent stroke model, possibly through upregulating antioxidant protein expression and maintaining mitochondrial coupling [135, 136]. Interestingly, resveratrol also provided a protective effect in an experimental

Fig. 3. The structure of curcumin.

recurrent stroke model through regulating AMP-activated protein kinase (AMPK) and SIRT1 signaling [137, 138].

Though not yet confirmed in a stroke model, resveratrol scavenges ONOO[−] and inhibits protein nitration in several other models [139–141]. Moreover, liquid chromatography with tandem electrospray mass spectrometry showed that resveratrol is a substrate of ONOO[−]. It could directly scavenge ONOO[−] and reduce the authentic ONOO[−]-mediated bovine serum albumin nitrotyrosine, with an EC₅₀ value of 22.7 mM [142]. In a rat MCAO model, resveratrol treatment at reperfusion increased eNOS expression while decreasing detrimental iNOS expression in ischemic brains [143]. In vitro, resveratrol inhibited iNOS expression in LPS-treated mouse glial cells, subsequently reducing NO production [144]. Consistently, resveratrol inhibited iNOS expression and NO production in cortical neurons under oxygen glucose deprivation condition [127]. Inhibition of iNOS could reduce the overproduction of NO, which may eventually contribute to a reduction of ONOO[−].

Resveratrol inhibited MMP-9 expression and activity in rodent ischemic brains within 24 h after MCAO, subsequently reducing BBB damage and brain edema [145, 146]. A recent study adopting a molecular docking strategy showed that resveratrol directly occupied the active site of MMP-9 through interacting with the Glu 402, Ala 417 and Arg 424 residues of MMP-9 [147]. This result explains how resveratrol directly inhibits MMP-9 activity in a stroke model. In addition, in vitro studies showed that inhibition of MMP-9 expression by resveratrol was possibly due to activation of peroxisome proliferator-activated receptor- α and inhibition of extracellular signal-regulated kinases (ERK1/2) [148, 149]. Those lines of evidence suggest that resveratrol is a potent MMP-9 inhibitor for ischemic stroke treatment.

Resveratrol also inhibited HMGB1 release in other systems possibly via suppressing the JAK/STAT1 signaling pathway [150, 151], which is important in mediating HMGB1 hyperacetylation and translocation [152]. Sirtuin-1 was recently identified as a novel mediator of deacetylation of HMGB1 [153]. Resveratrol could upregulate sirtuin-1, which may also contribute to inhibiting translocation of HMGB1 [154]. In addition to inhibiting translocation, resveratrol was also shown to decrease HMGB1 expression in an atopic dermatitis mouse model [155]. However, whether resveratrol inhibits HMGB1 in a stroke model is still unknown yet and merits further investigation.

Curcumin

Curcumin is a polyphenolic compound derived from the Chinese medicine *Curcuma longa* Linn [156]. The structure of curcumin is shown in figure 3. Notably, curcumin treatment reduced HT of cerebral ischemia in diabetic rats [157]. Curcumin treatment after focal cerebral ischemia significantly and dose-dependently reduced infarct volume, decreased brain edema and BBB damage, improved the neurological outcome and reduced the mortality rate, with a therapeutic time window up to 4 h [158–160]. Curcumin exhibited antiapoptotic, antioxidative and anti-inflammatory effects in cerebral ischemia, which accounted for attenuated cerebral injury [160, 161]. It was shown that the antioxidative effect of curcumin was

Fig. 4. The structure of apocynin.

at least partially due to the activation of Nrf2 [162, 163]. Curcumin also decreased MMP-9 and TNF- α expression in ischemic brains [164]. As a nonspecific NF- κ B inhibitor, curcumin seems to inhibit MMP-9 via downregulating NF- κ B activity [165].

Curcumin reduced ONOO[−] levels and tyrosine nitration in ischemic brains [166]. Curcumin attenuated rat spiral ganglion neuron apoptosis induced by ONOO[−] and protected from mitochondrial oxidative stress [167]. It also prevented the cerebral vascular endothelial damage induced by the ONOO[−] donor 3-morpholinosydnonimine (SIN-1) [158]. A direct interaction between curcumin and ONOO[−] was observed with spectroscopic techniques [168]. Curcumin could also inhibit iNOS and reduce the nitrite/nitrate content in LPS- or TNF- α -treated astrocytes in vitro [158]. Consistently, curcumin reduced the nitrite/nitrate level in vivo in a rat thromboembolic model [159].

Curcumin treatment reduced HMGB1 expression in a rat global cerebral ischemia reperfusion model [169]. Similarly, pretreatment with curcumin reduced HMGB1 expression in a cardiac ischemia-reperfusion injury model [170]. Curcumin inhibited HMGB1 release possibly via inhibiting acetylation of lysine residues [171]. Taken together, curcumin may target ONOO[−]/HMGB1/MMP-9 signaling cascades and has the potential to ameliorate HT in ischemic stroke with t-PA treatment.

Apocynin

Apocynin is derived from the medical plant *Picrorhiza kurroa* and other sources, and is an NADPH oxidase inhibitor [172]. The structure of apocynin is shown in figure 4. Apocynin treatment reduced the brain infarct size at 24 h after cerebral ischemia [173, 174]. Apocynin also attenuated the BBB damage in a hyperglycemic rat MCAO model, possibly through downregulating MMP-9/-2 and protecting tight junction proteins [175, 176]. More importantly, apocynin attenuated HT, protected BBB integrity and decreased infarct volume in hyperglycemic rats with t-PA treatment [177]. Apocynin inhibited NADPH oxidase and increased HDACs, and subsequently reduced HMGB1 release and cerebral ischemia damage [178]. Consistently, apocynin also reduced HMGB1 release in ethanol-treated SH-SY5Y cells and primary cortical neurons in vitro [179]. In addition to inhibiting HMGB1, pretreatment with apocynin upregulated bcl-2 expression, but downregulated bax expression, and subsequently inhibited cell apoptosis in a rat MCAO model. Apocynin also reduced proinflammatory cytokine IL-1 β in the same model [180]. Apocynin decreased the nitrotyrosine level in ischemic brains [180] as well as other experimental systems [181–186]. The effects of apocynin on scavenging O₂[−] in ischemic brain tissues were also observed in a rat transient focal cerebral ischemia model, which was related to inhibiting the NADPH oxidase isoform Nox2 [173, 187]. The inhibition or scavenging effects of apocynin on O₂[−] may account for reduced ONOO[−] production in ischemic brains. It is worth mentioning that apocynin treatment increased the mortality rate in an aged rat MCAO model and failed to reduce the infarct volume and BBB damage [188]. Moreover, apocynin treatment at high dosage increased HT in a transient MCAO mouse model [189]. These results suggest that the effects of apocynin on

Fig. 5. The structure of glycyrrhizin.

Fig. 6. The structure of caffeic acid.

ischemic stroke depend on the age of the subject as well as treatment dosage, and further research should be carefully conducted to address these issues.

Glycyrrhizin

Glycyrrhizin is a main constituent of herbal medicine *Glycyrrhiza glabra*, which is used in 60% of TCM formulas [190]. The structure of glycyrrhizin is shown in figure 5. Glycyrrhizin is a well-known HMGB1 inhibitor. Surface plasmon resonance analysis revealed that glycyrrhizin bound to HMGB1 with a moderate equilibrium dissociation constant value and reduced the interaction of HMGB1 and its receptor RAGE in a traumatic brain injury model [191]. Glycyrrhizin reduced HMGB1 secretion in ischemic brains and downregulated inflammatory factors, including TNF- α , iNOS, IL-1 β and IL-6 [84, 192]. An in vitro study showed that glycyrrhizin inhibited the PKC and calcium/calmodulin-dependent protein kinase IV activity in active microglia, subsequently reducing the phosphorylation of HMGB1 and its translocation, resulting in suppression of cytokine expression [192]. Glycyrrhizin also reduced the brain infarct volume and improved neurological outcomes both in a transient ischemic stroke model and an acute hyperglycemia stroke model [84, 192, 193]. Moreover, glycyrrhizin exhibited antiapoptotic effects via inhibiting IL-17A-mediated reduction of bcl-2/bax ratio and cytochrome c release in ischemic brains [84, 194]. Glycyrrhizin also reduced oxidative stress and NF- κ B-related brain inflammation in rodent ischemic stroke [195].

Though not yet confirmed in a cerebral ischemia model, glycyrrhizin reduced nitrotyrosine in a gut ischemia-reperfusion model [196], carrageenan-induced lung injury in mice [197] and spinal cord compression injury in mice [198]. The inhibitive effects of glycyrrhizin on MMP-9 were also observed in several studies [199, 200]. Therefore, glycyrrhizin could be a promising candidate for a combined therapy for reducing HT induced by delayed t-PA treatment, possibly via targeting ONOO[−]/HMGB1/MMP-9 signaling cascades.

Caffeic Acid

Caffeic acid is a phenolic compound found in many medicinal plants [201]. The structure of caffeic acid is shown in figure 6. Caffeic acid treatment significantly and dose-dependently attenuated hippocampal neuron loss and improved the motor function of rats in a global

Fig. 7. The structure of tanshinone IIA.

cerebral ischemia model via inhibiting 5-lipoxygenase and NF- κ B [202]. Similarly, caffeic acid improved neural viability and synapses, and attenuated memory deficit in a permanent MCAO mouse model [203]. More importantly, caffeic acid treatment for 5 days after ischemia significantly reduced rat brain infarct volume and inhibited astrocyte proliferation at day 14 in a transient MCAO model, indicating the long-term efficacy of caffeic acid [204]. Caffeic acid scavenged ONOO[−] and reduced ONOO[−]-mediated tyrosine nitration [205]. In addition, caffeic acid is also an MMP-9 inhibitor with an IC₅₀ value of 10–20 nM [206]. Hence, caffeic acid could possibly target ONOO[−]/HMGB1/MMP-9 signaling cascades in ischemic stroke to attenuate HT induced by delayed t-PA treatment.

Tanshinone IIA

Tanshinone IIA is one of the active ingredients of *Salvia miltiorrhiza* root. The structure of tanshinone IIA is shown in figure 7. Tanshinone IIA demonstrated an antiapoptotic effect via upregulating bcl-2 expression and downregulating active caspase-3 in the cortex, subsequently reducing neural apoptosis, decreasing the brain infarct volume and attenuating neurological deficit in a rat MCAO model [207, 208]. In addition, tanshinone IIA scavenged RNS in an ischemic stroke model [209]. Tanshinone also exhibited an anti-inflammatory effect through reducing macrophage migration inhibitory factor and proinflammatory cytokines such as IL-6 and TNF- α in a rat MCAO model [210]. Tanshinone IIA treatment prevented BBB damage and brain edema via suppressing MMP-9 expression, reducing HMGB1 levels and NF- κ B activation and protecting tight junction proteins in rat transient MCAO models [211, 212]. Consistently, tanshinone treatment at reperfusion for 15 days inhibited HMGB1 expression and NF- κ B activation, protecting from neuron apoptosis in a rat MCAO model [213]. Inhibition of HMGB1 by tanshinone IIA was also found in a myocardial infarction model, coinciding with attenuated inflammation and oxidative stress [214]. Taken together, tanshinone IIA could protect against ischemia stroke injury and targets HMGB1, MMP-9 and free radicals. Hence, tanshinone IIA is a promising compound for mitigating ischemic stroke injury and attenuating HT induced by t-PA treatment.

Discussion

In this review article, we have discussed the important roles of ONOO[−]/HMGB1/MMP-9 signaling cascades in mediating neural cell death, infarction enlargement and BBB damage, and their potential roles in mediating HT induced by delayed t-PA treatment. These three factors may work together as a pathway significantly contributing to HT. Thus, targeting ONOO[−]/HMGB1/MMP-9 signaling cascades could be an important strategy to reduce HT. Natural compounds from Chinese medicine including baicalin, resveratrol, curcumin, apocynin, glycyrrhizin, caffeic acid and tanshinone IIA have shown the potential to be combined therapies with t-PA and prevent t-PA-mediated HT and ischemic brain injury

through targeting ONOO[−]/HMGB1/MMP-9 signaling cascades. For example, apocynin ameliorated HT in hyperglycemic rats [177]. Similarly, curcumin inhibited HT in a diabetic rat cerebral ischemia model [157]. These studies suggest that apocynin and curcumin have a high potential for further development into adjunct therapies with t-PA. Nevertheless, it has not yet been tested whether most of the other selected compounds could help reduce HT in ischemic stroke with thrombolytic treatment. Although further studies are needed to verify their efficacies in reducing HT, the selected compounds are good candidates for developing adjunct therapies with t-PA, not only because of their efficacies in ameliorating ischemic brain injury and HT but also their good safety profiles. For example, baicalin is an active ingredient in flavocoxid, an FDA-approved medicinal healthy food [15]. Resveratrol is well tolerated by human, as suggested by a recent phase II clinical trial, with the highest dosage of 1,000 mg twice daily [215]. Similarly, curcumin is also safe for human at a dosage of 1,000 mg daily for 6 weeks [216]. The high tolerance of those compounds in human further makes them potential candidates for translational study.

Nevertheless, the direct effects of those compounds on the ONOO[−]/HMGB1/MMP-9 signaling cascades remain to be further addressed. Firstly, whether the selected compounds could target all these three molecules and improve the therapeutic outcome is still unclear. For example, though resveratrol was shown to inhibit HMGB1, studies demonstrating its effects on a stroke model are still lacking. A similar situation applies to other compounds. Secondly, whether the compounds can directly modify the targets needs further investigation. Some studies showed the direct interaction of the compounds with the targets. For example, resveratrol directly binds to the active site of MMP-9, as revealed by a molecular docking study [147], and baicalin directly scavenges ONOO[−], as shown by a mass spectrum analysis [108]. However, most of the other studies only observed the phenomenon of the modulatory effects on these targets, and the exact modulatory processes are still unknown. Hence, whether those compounds have the direct effects on the targets or just a consequence of the indirect response to treatments with the compounds remains unclear and merits further investigation.

As for development of those compounds into adjunct therapy, following important issues should be considered. The first issue is the time point for outcome measurement. Most of the preclinical studies only evaluated the HT results within 24 h after cerebral ischemia [217]. However, HT could occur in both the early and the late phase of cerebral ischemia, even days after stroke [218]. Thus, measuring the HT results at 24 h is not good enough for thoroughly evaluating the effect of a combination therapy, as prevention of HT at an early stage does not necessarily ensure its protective effects at a late phase. Hence, the observation of HT may be extended to days after ischemia onset. The second issue is the therapy duration. As the measurement time point is extended to days after stroke, whether repeated treatment is more beneficial needs to be addressed further. Repeated treatment may continuously modulate the related targets and enhance the therapeutic effects on HT. On the other hand, treatment in a late phase may cause unfavorable outcomes due to the protective roles of those targets in a late phase of stroke. Evidence has shown that MMP-9 and HMGB1 may be beneficial in a late phase of stroke. In detail, MMP-9 contributes to the remodeling of neuroplasticity [219]. Inhibition of MMP-9 beginning on day 7 after cerebral ischemia increased brain injury and impaired the functional outcomes of rats at day 14 after MCAO [219]. Similarly, HMGB1 deprived of astrocytes was upregulated on day 14 after stroke, which promoted endothelial progenitor cell-mediated neurovascular remodeling [220]. Inhibition of HMGB1 with siRNA significantly reduced angiogenesis and worsened the neurological outcome [220]. Thus, targeting MMP-9 or HMGB1 with these compounds in a late phase may inhibit neurogenesis and angiogenesis, subsequently leading to unfavorable outcomes. Further investigations into the dynamic changes of ONOO[−], HMGB1 and MMP-9 and their roles in different phases may help to predict the optimal therapy duration. Some studies observed the long-

term protective effects. For example, Wang et al. [213] found that treatment with tanshinone IIA for 7 days or 14 days reduced the apoptosis in ischemic brains. However, they did not evaluate the optimal therapy duration. The optimal duration of treatment with these natural compounds should be determined by using different treatment durations with a relatively long observational period.

In addition to therapy duration and the time of outcome measurement, treatment dosage and dose-dependent responses should also be determined in further investigations. Although current studies have identified the dose-dependent response to these compounds, it is necessary to reevaluate the optimal dosage for a combination therapy with t-PA, as the outcome involves HT, which has not been investigated before. Potential drug-drug interactions of these compounds with t-PA should also be carefully examined in the development of combination therapy. To determine whether these compounds affect the thrombolytic effect of t-PA, it is necessary to adopt the thromboembolic model rather than the MCAO model for future studies [217].

Conclusion

ONOO[−], HMGB1 and MMP-9 are important pathological factors in cerebral ischemia-reperfusion injury. The interaction of ONOO[−], HMGB1 and MMP-9 can form ONOO[−]/HMGB1/MMP-9 signaling cascades, potentially mediating the delayed t-PA-induced HT. Natural compounds from Chinese medicine including baicalin, resveratrol, curcumin, apocynin, glycyrrhizin, caffeic acid and tanshinone IIA are promising drug candidates for alleviating t-PA-mediated HT possibly via inhibiting the ONOO[−]/HMGB1/MMP-9 signaling cascades in ischemic stroke. Further studies are warranted to evaluate these compounds as a combination therapy to reduce HT in ischemic stroke with t-PA treatment.

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Disclosure Statement

The authors declare no conflict of interest.

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