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Interaction of free radicals, matrix metalloproteinases and caveolin-1 impacts blood-brain barrier permeability

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1. ABSTRACT

Free radicals play an important role in cerebral ischemia-reperfusion injury. Accumulations of toxic free radicals such as reactive oxygen species (ROS) and reactive nitrogen species (RNS) not only increase the susceptibility of brain tissue to ischemic damage but also trigger numerous molecular cascades, leading to increased blood-brain barrier (BBB) permeability, brain edema, hemorrhage and inflammation, and brain death. Activating matrix metalloproteinases (MMPs) is a key step in BBB disruption. MMPs are proteolytic zinc-containing enzymes responsible for degradation of the extracellular matrix around cerebral blood vessels and neurons. Free radicals can activate MMPs and subsequently induce the degradations of tight junctions (TJs), leading to BBB breakdown in cerebral ischemia-reperfusion injury. Recent studies revealed that caveolin-1, a membrane integral protein located at caveolae, can prevent the degradation of TJ proteins and protect the BBB integrity by inhibiting RNS production and MMPs activity. The interaction of caveolin-1 and RNS forms a positive feedback loop which provides amplified impacts on BBB dysfunction during cerebral ischemia-reperfusion injury. Here, we reviewed the recent progress in the interactions of RNS, caveolin-1 and MMPs. Current evidence indicates that the interactions of RNS, caveolin-1 and MMPs are critical signal pathways in BBB disruption and infarction enlargement during cerebral ischemia-reperfusion injury.

2. INTRODUCTION

The blood-brain barrier is a physical barrier which protects the brain milieu and consists of endothelial cells of the microvessels, astrocytic endfeet and extracellular matrix (ECM). Among these three components, the layer of endothelial cells is the key barrier due to the formation of tight junctions. The primary responsibility of the BBB is the strict regulation of trans-BBB permeability. In this regard, the endothelial TJs of the capillary are the main mediators, limiting transport of vascular derived substances. The BBB breakdown occurs in many neurological diseases, including stroke, meningitis, encephalitis, trypanosomiasis, multiple sclerosis, neuromyelitis optica, Alzheimer's disease, brain carcinoma, and so on. During pathological conditions, the BBB permeability is regulated by highly specialized proteins, which can be modulated by many intracellular and extracellular signaling pathways.

Free radicals including ROS and RNS are important pathological mediators in stroke (See reviews (1-3)). The BBB opening after cerebral ischemia is often associated with the productions of ROS and RNS (4). Pathological pathways leading to the BBB dysfunction include macrophage/microglial activation, neutrophil recruitment, mitochondrial insults and excitotoxicity, all of which converge on the same point: overload of free radicals. Oxidative damage triggered by accumulated free radicals

induces many downstream pathways that mediate the activation of matrix metalloproteinases and tight junction protein degradation, subsequently resulting in the BBB hyper-permeability in the ischemic brain injury (3, 4).

Notably, as the main components of RNS, peroxynitrite and its decomposition products are of especial interest in oxidative damage and BBB dysfunction during cerebral ischemia/reperfusion (I/R) injury. Peroxynitrite is formed by the reactions of nitric oxide (NO) and superoxide. Elevated levels of RNS and tyrosine nitration were seen in brain sections of stroke patients and in experimental ischemic animal models. Moreover, superoxide scavengers, nitric oxide synthase (NOS) inhibitors and peroxynitrite decomposition catalysts (PDCs) attenuated BBB disruption, reduced infarction volume and improved neurological dysfunctions in cerebral I/R injury (5, 6).

The molecular mechanisms of the RNS-induced BBB breakdown are largely unclear yet. Recent evidence indicates that caveolin-1 (cav-1), a membrane embedded protein which lies specifically in caveolae, appears to participate in the modulation of oxidative damage during cerebral ischemia-reperfusion injury. For example, caveolin-1 knockout mice had an enlargement of infarction volume in an experimental stroke model (7). Cav-1 could regulate the BBB permeability evidenced by cav-1 directly binding to matrix metalloproteinases (MMPs), and inhibiting MMPs activity (8). There is an inter-regulatory relationship in the interaction of cav-1 and NO. On one side, NO modulates cav-1 expression at both mRNA and protein levels (9); on the other side, cav-1 inhibits the activity of all three isoforms of NOS thereby affecting NO production (10). Thus, the interaction of caveolin-1 and RNS may function as a signal pathway in modulating the BBB permeability during cerebral ischemia-reperfusion injury.

In this review, we mainly focus on the roles of oxidative stress in BBB disruption, the interactions of free radicals, MMPs and caveolin-1 and their impact in regulating the BBB permeability during cerebral ischemia-reperfusion injury. We first review the factors involved in the BBB breakdown during the ischemic stroke; next we discuss the interactions of caveolin-1 and free radicals in the regulation of BBB permeability. In particular, the contributions of RNS to the BBB opening have been highlighted in depth. Finally, we summarize the genetic associations of caveolin-1 and RNS in the endothelial cells.

3. ROLE OF MATRIX METALLOPROTEINASES IN REGULATING BBB PERMEABILITY DURING CEREBRAL ISCHEMIA-REPERFUSION INJURY

The primary causes of mortality following ischemic stroke are brain edema and hemorrhagic transformation. The common pathophysiological pathway leading to these complications is the disruption of the BBB. Loss of glucose and oxygen during brain ischemia brings about a cascade of events including depletion of ATP, glutamate excitotoxicity and subsequent calcium influx, acidosis, oxidative and nitrosative stress, and enzymatic activation. Obvious changes in BBB permeability during cerebral ischemia may not occur immediately, requiring several hours of continuous occlusion and/or reperfusion to induce a measurable increase in paracellular permeability (11). During ischemic stroke and subsequent reperfusion, the TJs of the BBB are degraded, resulting in the influx of substances into brain parenchyma from blood vessels. Activation of proteases, such as MMPs, cathepsins, heparanase, and tissue plasminogen activator (t-PA), induces the degradations of the BBB extracellular matrix and tight junction proteins, consequently leading to BBB opening and brain vasogenic edema (12). The activated enzymes may further induce cell death through integrin-mediated mechanisms (13, 14). Furthermore, early after the onset of ischemia, MMPs contribute to the disruption of the BBB, leading to vasogenic edema and the influx of leucocytes into the central nervous system (CNS). Mounting new MMP family members, their substrates and mediators were found during past decades. They play important roles in the BBB insults (12, 15-17). Herein, we summarized the roles of MMPs in the degradations of tight junction proteins and extracellular matrix during BBB breakdown.

3.1. Tight junction proteins and the basal lamina

The primary defense between the systematic circulation and brain parenchyma is the capillary endothelium. The difference between the endothelial cells in the BBB from those in others organs is the presence of the tight junction complex (17), including transmembrane components and cytoplasmic accessory proteins. TJs are present within the intercellular cleft, together with surrounding adherent junctions (AJs) to form a zipper-like seal between adjacent endothelial cells. This blocks the paracellular flux of hydrophilic and large molecules across the BBB (18). Under physiological circumstances, most hydrophilic molecules and large hydrophobic substances cannot cross the BBB freely (19).

The restriction barrier of the BBB is governed by three essential transmembrane components including junctional adhesion molecule-1 (JAM-1) (20), occludin, and claudins. The cytoplasmic domains of these three proteins are anchored to the cytoskeleton through accessory proteins like zonula occludens (ZOs) family. JAM-1, a 40-kDa protein belonging to the IgG superfamily, has a single membrane-spanning chain with a large extracellular domain (21). Loss of the BBB integrity is correlated with decreased expression of JAM-1 (22). Occludin, a 60- to 65-kDa phosphoprotein, has four transmembrane domains whose carboxyl and amino terminals are located in the cytoplasm and two extracellular loops that span the intercellular cleft (23). The cytoplasmic C-terminal domain is likely involved in the association of occludin with the cytoskeleton via accessory proteins like ZO-1 and ZO-2 (24, 25). Over-expression of wild type, truncated C-terminus and multiple domains of occludin induced increased transcellular electrical resistance and decreased paracellular permeability (26, 27). Claudins, a group of 20- to 24-kDa proteins, have two extracellular loops that interlink with other claudins in adjacent endothelial cells to form the primary seal of the TJs. The internal loops of the claudins

can bind to ZO-1, ZO-2, and ZO-3 via their carboxy-terminals (28). Among the claudin family members, claudin-3, -5, and -12 had been identified in the endothelial cells of the BBB. Claudin-5 is specifically involved in the active regulation of paracellular transport of small molecules; permeability to large molecules was enhanced in claudin-5 null mice (29). ZOs have three domains including three PDZ, SH3 and guanylyl kinase-like domain. These protein-protein interaction domains serve as a scaffold for other proteins in the membranes. The proline-rich carboxyl-terminus of the ZOs can bind to actin and anchor other TJ proteins to the actin cytoskeleton (25).

In addition, brain endothelial cells are surrounded by the basal lamina, a layer of ECM. The basal lamina is mainly composed of structural proteins including collagen type-IV, fibronectin, laminin, etc. It functions to connect and separate endothelial cells and astrocytes from one another, regulating intercellular signaling. The basal lamina is essential for keeping the integrity of the BBB. During ischemia and reperfusion, the degradation of proteins on the basal lamina leads to the increased BBB permeability (30, 31). During cerebral I/R injury, proteolytic enzymes can mediate the degradation and dissolution of the basal lamina components, inducing the detachment of astrocytes and endothelial cells and the microvessels collapse and resulting in the BBB leakage.

3.2. Matrix metalloproteinases

A host of evidence has shown that MMPs activation is the main cause of BBB breakdown through the degradation of tight junction proteins and ECM components (12, 16, 32). MMPs, of which there are more than 20 members, belong to a family of Zn^{2+} and Ca^{2+} dependent endopeptidases (33, 34). They have three domains: an N-terminal propeptide, a catalytic domain and a hemopexin-like C-terminus. Apart from a subclass of membrane-bound MMPs (MT-MMPs), most of them are secreted in the interstitial space as an inactive zymogen and are activated by other regulatory proteins. Clinical and experimental evidence revealed that MMPs were elevated in the brains of stroke patients and ischemic model animals (35, 36). In the MMP family, MMP-2 and MMP-9 appear to play pivotal roles in BBB opening during cerebral ischemia-reperfusion injury due to their substrate specificity. Both of them are secreted as inactivated zymogens. Pro-MMP-2 can be activated by MT-MMP (37) and inactivated by tissue inhibitor of metalloprotease-2 (TIMP-2) (38). During cerebral ischemia, the activation of MMP-2 was found at the first stage of the biphasic BBB opening (39-41). Enhanced MMP-9 activity was related to the second stage (32, 42). The activation of pro-MMP-9 by MMP-3 and the accumulation of ROS were simultaneously found in ischemia-reperfused brains (43). Reperfusion following cerebral ischemia injury further accelerates the activations of MMP-2 and MMP-9 (44). In this process, the activation of MMP-9 is crucial in the disruption of TJ proteins and BBB permeability. MMP-9 knockout mice had increased ZO-1 level, decreased BBB permeability and reduced brain edema in experimental stroke model (45). Besides, other experimental evidence have also revealed that the ischemia-reperfused rat brains had increased activities of MMPs and reduced levels of claudin-5 and occludin whereas treatment of an MMP inhibitor restored these TJ proteins, indicating that MMPs also mediate the degradation of the TJ proteins (41, 46).

4. OXIDATIVES STRESS INDUCES BBB LEAKAGE VIA ACTIVATING MMPs PATHWAY

Free radicals (including ROS and RNS) are produced under both physiological and pathological conditions. Low concentrations of free radicals appears to function as secondary messengers, activating complicated signal pathways and regulating cell proliferation, differentiation, apoptosis, synaptic signal transmission and plasticity. On the other hand, free radicals at high concentrations are important pathological mediators occurring in most ischemic and neurodegenerative diseases (47). Free radicals can attack proteins, DNA and lipids and bring detrimental effects for living organisms (see review (1, 2)).

Free radicals have been implicated in ischemic stroke and serve as an important contributor to cell and tissue injury (48). During acute cerebral ischemia-reperfusion injury, the sudden disruption of oxygen and reoxygenation results in the production of free radicals that lead to “reperfusion injury”. Ischemia-reperfusion insults impair the functions of redox systems and disable the brain’s ability to remove excessive free radicals, consequently aggravating tissue oxidative damage in ischemic brains (3, 48). The BBB is one of the important targets in free radical-induced brain damage during cerebral ischemia-reperfusion injury. In most cases, ROS and RNS are simultaneously produced, and their reactions and transforms are extremely fast. Due to technical limitations, it is difficult to clearly delineate the precise mechanisms of ROS and RNS in BBB disruption.

4.1. ROS and BBB leakage

During ischemia-reperfusion injury, an oxygen burst produces a large amount of ROS, including superoxide, hydroxyl radicals etc., that overwhelm endogenous scavenging systems to form oxidative stress and modify cellular macromolecules. ROS are mostly produced from mitochondria during electron transport (3). In the ischemic brain, ROS can induce MMPs activation, endothelial TJs degradation, macromolecular damage, and result in BBB leakage (4, 49, 50). Because of its highly reactive characteristic, ROS can oxidize and peroxidize many biomolecules such as proteins and lipids, which, in turn, affect BBB permeability. For instance, membrane lipid peroxidation can directly influence BBB integrity. 4-hydroxynonenal, a toxic lipid peroxidation product, stimulated BBB opening in an *in vitro* BBB model (51). Inhibition of lipid peroxidation induced by arachidonic acid (AA) decreased BBB permeability (52). Glutathione depletion increased the severity of membrane protein damage and BBB leakage in both adult and aged gerbils (53).

Several major pathways are associated with the production of ROS in the ischemia insults, including

excitotoxicity, neutrophil recruitment, and mitochondrial dysfunction (4). Some of them may share the same key regulators that affect the ROS generating network. During ischemia-reperfusion injury, neutrophils are recruited to the BBB as a result of the inflammatory response (54), ROS are produced by activation of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase in infiltrated neutrophils (55, 56). Ischemia-reperfusion activates NOS and enhances NO production. Simultaneously ischemia-reperfusion stimulates NADPH oxidase and generates superoxide anions, subsequently producing peroxynitrite (57, 58). Both ROS and RNS can activate MMPs, target TJs and ECM proteins and affect BBB permeability.

ROS can also induce and/or activate MMPs. Superoxide dismutase (SOD)-2 knockout mice had decreased BBB integrity and the increased MMPs activity (59). SOD-1 transgenic mice had reduced MMP-9 activity, lesion size and brain edema in the forebrain (60). On the other hand, knockdown of NADPH oxidase decreased the ROS production and blocked the upregulation of MMP-9 at both protein and mRNA levels by regulating NF-κB-dependent MMP-9 promoter activity (61). Similarly, inhibition of NADPH oxidase with apocynin decreased the activation of MMP-9, whereas normobaric hyperoxia reduced the expression of NADPH oxidase subunit and MMP-9 activity in isolated rat brain microvessels after middle cerebral artery occlusion (MCAO) (62). In addition, MMP-2 was activated by treatment of xanthine/xanthine oxidase which produced superoxide and hydroxyl radicals in cultured smooth muscle cells (63). However, as the authors have pointed out, the activation of MMP-2 could be due to the enzyme itself rather than the ROS generation, because xanthine/xanthine oxidase-induced proMMP-2 mediated activation could not be inhibited after treatment with both SOD and catalase (63).

The degradation, down-regulation, modification and mislocalization of TJs proteins directly induce BBB hyperpermeability. ROS can degrade TJ proteins via activating MMPs. It is reported that ROS produced from xanthine/xanthine oxidase system resulted in redistribution and degradation of occludin and claudin-5 through acting on Rho, PI3K and PKB pathways (64). Besides, treatment with hydrogen peroxide (H_2O_2) decreased transendothelial electric resistance (TEER) through redistribution of occludin and ZO-1 to non-TJs in primary cultured bovine brain microvascular endothelial cells (BMECs) (65). Superoxide can induce a rapid increase of tyrosine phosphorylation and dissociate occludin-ZO-1 complex from cytoskeleton mediated by Src (66). Hydrogen peroxide can also induce the mislocalization of ZO-1, ZO-2 and occludin in cultured porcine BMECs (67) and Caco-2 cell monolayers (68). A recent study showed a time-dependent reduction of occludin with an increase of superoxide in the rat brain with obstructive jaundice (69). Therefore, TJs proteins degradation may be the main cause of ROS-induced BBB opening during cerebral ischemia-reperfusion injury.

4.2. RNS and BBB leakage

Nitric oxide and peroxynitrite ($ONOO^-$) are major components of RNS. In many cases, NO is enzymatically generated from the conversion of L-arginine and oxygen through nitric oxide synthase. There are three isoforms of NOS that produce NO in living organism with L-arginine as substrate: neuronal NOS (nNOS, type 1), inducible NOS (iNOS, type 2) and endothelial NOS (eNOS, type 3) (See review (70)). Among them, eNOS and nNOS subtypes are calcium dependent enzymes and are constitutively expressed in almost all cell types. The activations of eNOS and nNOS generally produce a small amount of NO (at nanomole level) (71, 72), but the iNOS subtype is calcium independent and produce large amounts of NO (at micromole level) (73). iNOS is often activated on a transcriptional level by de novo synthesis in response to many stimulating agents (74). Physiological concentrations of NO (at levels less than 10 nM) generated from eNOS are essential in neuronal communication, regulation of vascular tone, synaptic transmission, platelet aggregation and inflammation response (75-79). During cerebral ischemia, however, high concentrations of NO generated by calcium-dependent nNOS activation and the activation of iNOS by macrophages and other cell types are detrimental to the ischemic brain. High concentrations of NO can induce inflammation and cell death, resulting in enlargement of infarction size ((9, 80-83). In addition, during ischemia-reperfusion insults, NO and superoxide are produced simultaneously and react to form peroxynitrite ($ONOO^-$). This reaction is determined by concentration of NO and superoxide which are controlled by the activity of NOS and SOD, respectively (84, 85). NO, peroxynitrite and their derivatives generate nitrosylative stress that cause detrimental effects in ischemia-reperfused organs (86).

4.2.1. NO and BBB leakage

NO is a critical small molecule in the BBB disruption during cerebral ischemia-reperfusion injury. Treatment of nonselective NOS inhibitor Nώ-nitro-L-arginine (L-NAME) dramatically decreased infarction volume and BBB leakage in several experimental cerebral ischemic animals (5, 6). The protective and detrimental effects of NO on the ischemic brains are dependent on the concentration of NO produced from different NOS subtypes. The different isoforms of NOS appear to be activated at different time windows during cerebral ischemia-reperfusion injury (70). Using electron paramagnetic resonance spin trapping technology, we directly trapped and detected the production of NO from ischemia-reperfusion rat brains and found a biphasic increase of NO in both the ischemic core and penumbra of the ischemia-reperfused rat brains with the first phase at the 1 hr of ischemia and the secondary phase at 24 to 48 hrs of reperfusion after 1hr ischemia. The first and secondary phase of NO production correlated with increased nNOS and iNOS respectively (9). More supportive evidence has been obtained from the mice with different NOS subtype deficiencies. After MCAO, mice deficient in eNOS had larger infarction volumes than in the wild type mice (87), whereas nNOS knockout mice had smaller infarction sizes and less brain edema (88, 89). Administration with anti-serum of nNOS in early traumatic brain injury mice significantly ameliorated BBB leakage, edematous swelling and sensory motor disturbances (90). Interestingly, in male mice with MCAO ischemia, iNOS null mice had lower

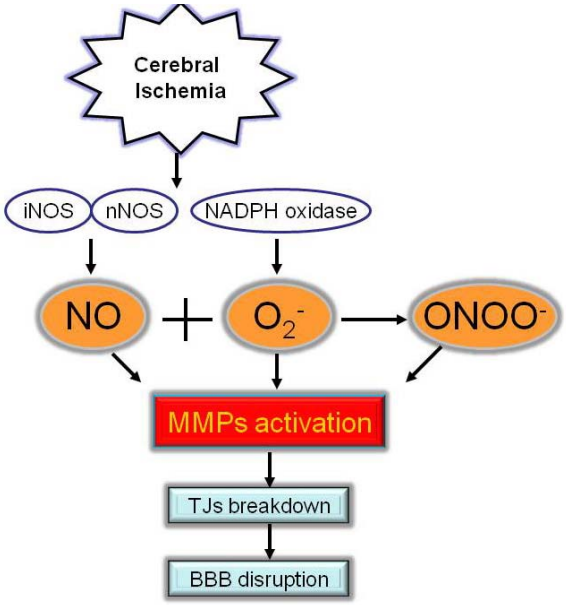

Figure 1. Formation of peroxynitrite and the role in MMPs-induced TJs and BBB breakdown during cerebral ischemia and reperfusion insults. Upon onset of ischemia and after several hours of reperfusion, superoxide (O_2^-) and nitric oxide (NO) are produced by nicotinamide adenine dinucleotide phosphate (NADPH) oxidase and nitric oxide synthase (NOS), respectively. Peroxynitrite, formed by the reaction of superoxide and NO, activates MMP-2 and 9 and digests tight junction proteins, leading to disruption of the TJs and BBB.

infarction volumes than the wild type mice, but this was not the case in female mice (91). Similar responses were found in male and female mice treated with the iNOS inhibitor aminoguanidine. These results indicate that the there is a gender difference in the role of iNOS in ischemic brain injury (91).

Current progress suggests that the activation of MMPs is one of the critical pathways in NO-induced cell death. NO can negatively regulate MMPs activities in various cells. Treatment with an NO donor or transfection of eNOS vector down-regulated the expression of MMP-2 mRNA in endothelial cells dose-dependently (92). In cultured aortic smooth muscle cells, the inhibition of NOS expression led to a dose-dependent increase in the expression of MMP-9 (93). Similar results were obtained in tumor cells or tumor/endothelial cells (94, 95). In rat renal mesangial cells, NO was revealed to reduce the stability of MMP-9 mRNA (96). The activation of MMP-9 is also associated with the expression of nNOS. The expression of nNOS was colocalized with the activated MMP-9 in the ischemic mouse brain. MMP-9 activity was attenuated in the mice treated with nNOS selective inhibitor or in nNOS null mice (97). The NO-induced S-nitrosylation contributes to the activation of MMP-9 and neuronal cell death during cerebral ischemic injury (97). Therefore, the protective or detrimental roles of NO are highly dependent on cell type, the specific environment, and the amount of NO produced.

4.2.2. Peroxynitrite and BBB leakage

The formation of peroxynitrite ($ONOO^-$) is required or even decisive in the process of NO-induced MMPs activation and BBB breakdown. Peroxynitrite is formed by the reaction of NO and superoxide and further decomposed into a series of other cytotoxic reactive species, such as nitrogen dioxide ($\bullet NO_2$), dinitrogen trioxide (N_2O_3) and peroxynitrous acid (ONOOH), as well as other free radicals (hydroxyl and carboxyl radicals) (98). Peroxynitrite and its derivates are membrane permeable molecules that can easily penetrate the cell membrane (99). Peroxynitrite itself is more permeable through lipid membrane and has higher cytotoxicity than superoxide (99, 100). Peroxynitrite and its decomposers are highly reactive intermediates that induce the nitration of tyrosine and cysteine residues in proteins and subsequently modulate the proteins during cerebral ischemia and reperfusion insult (101). They induce hydroxylation, peroxidation and nitration of the proteins and nucleotides, break DNA structure, and bring the damages to cell function or even lead to cell death (85, 102). The DNA strand breaks then lead to the activation of the DNA repair enzyme, poly (adenosine diphosphate-ribose) polymerase (PARP), which uses NAD+ as a substrate. Massive DNA damage mediated by I/R injury results in excessive PARP activation, leading to depletion of its substrate NAD+ and ATP and eventually to apoptosis; this is also known as peroxynitrite-PARP pathway induced cell death (103).

Mounting evidence indicates that peroxynitrite is detrimental to the BBB. Oxygen-glucose deprivation or ischemia-reperfusion induces $ONOO^-$ production in neurons and brain vascular endothelial cells (104-106). Increased 3-nitrotyrosine (3-NT, an $ONOO^-$ oxidative product) was found in the ischemic penumbral cortex (6, 107). By co-localizing 3-NT, MMP-9 and Evans blue leakage, a recent study suggests that $ONOO^-$ is associated with MMP-9 activation and BBB opening in focal cerebral ischemia-reperfusion rats (6). Treatment of L-NAME inhibited the production of 3-nitrotyrosine decreasing MMP-9 activity and attenuating BBB permeability (5, 6). PDCs like FeTMPyP and FeTPPS can potentiate the reduction of NO and $O_2\cdot^-$, and isomerize $ONOO^-$ to nitrate and decrease its decomposition to other reactive intermediates. Even delayed treatments of FeTMPyP and FeTPPS at 2 and 6 h after MCAO can reduce apoptosis, infarction volume, edema and neurological deficits in focal ischemic rat brains. The neuroprotective effects of PDCs are associated with the reduction of $ONOO^-$ in blood and brain tissues (108). The effects of FeTMPyP on the infarction volume and neurological defects were even better when FeTMPyP was combined with PARP inhibitor rather than FeTMPyP alone in a focal cerebral ischemia model (109). The effects of FeTMPyP on the BBB integrity were also reported in an *in vitro* BBB model (110).

Peroxynitrite can activate MMPs and induce the degradation of TJ proteins, subsequently destroying BBB integrity (See Figure 1). 3-morpholinosydnonimine (SIN-1, a peroxynitrite donor) instead of S-nitroso-N-acetyl-l,l-penicillamine (SNAP, an NO donor) increased the secretion of activated MMP-2 and expression levels of MT1-MMP

through the activation of NF-κB in a satellite hepatic cell line L190 cells (111). Treatment of the synthesized peroxynitrite at 50 μM directly inactivated TIMP-1. The effects were reversed by $ONOO^-$ scavengers such as thiourea, deferrioxamine, tyrosine, tryptophan and methionine (112). The synthesized peroxynitrite at higher concentrations (500 μM to 5 mM) caused TIMP-1 protein fragmentation (112). The effects of $ONOO^-$ on the MMP-8 activation were 1000 times greater than NO (113). Peroxynitrite dramatically activated MMP-1, 8, 9 from purified human zymogens in the presence of glutathione (114), via S-glutathiolation of oxidized form of cysteine in the auto-inhibitory domain of the zymogen, a process that is likely to occur in oxidative stress conditions (115, 116). TIMP-4 could be inactivated by $ONOO^-$ to form nitration products in four tyrosine residues, subsequently activating MMP-2 in endothelial cells (117). The infusion of the synthesized peroxynitrite into heart caused rapid increase of released MMP-2 with 10 min (118). A recent report showed that FeTMPyP prevented MMPs activation and neurovascular injury after I/R insults (119). Peroxynitrite can break down and rearrange tight junction proteins inducing BBB disruption. An obvious derangement of ZO-1 was observed in the human umbilical vein endothelial cells incubated with $ONOO^-$, which was significantly attenuated by poly-(ADP-ribose) polymerase inhibitors (120). Treatments of coumarin derivatives and semamol with $ONOO^-$ displayed an inhibitory effect on the degradation of tight junction proteins and the BBB disruption in the diabetic rats (121, 122). Nevertheless, due to the technical limitations, direct evidence about the roles of $ONOO^-$ in MMPs activation, TJs degradation and BBB opening in ischemic brain injury is unavailable in current literature. More importantly, the molecular mechanisms of the ONOO- -induced MMPs activation remain unexplored.

5. CAVEOLIN-1 REGULATES BBB PERMEABILITY

Caveolae are small (50-100 nanometers) invaginations of the plasma membrane that form flask structures in many vertebrate cells, especially in adipocytes and endothelial cells (123). As integral membrane proteins, caveolins are the structural proteins of caveolae and are essential to caveolae structure formation. Caveolae are specialized lipid rafts and play important roles in endocytosis, oncogenesis and uptake of microorganisms (124, 125). There are three members in the caveolin family: caveolin-1, 2 and 3.

Caveolins physically interact with a large number of proteins via the caveolin scaffold domain (CSD) (126). The binding proteins contain the CAV-binding site: "φXφXXXXφ" or "φXXφXXXXφ" where φ is Phenylalanine, Tyrosine or Tryptophan and X is any amino acid residue. Proteins with these character domains include eNOS, iNOS, nNOS, MMP-2, EGF receptor, and aquaporin, among others. Thus, caveolins can bind and regulate these proteins and participate in multiple cellular activities including mitogenic signaling (127), apoptosis (128, 129), cholesterol transport (130), cancer progression and metastasis (131, 132), and vascular diseases (133). Current evidence indicates that caveolin-1 (cav-1) is a critical protein in the regulation of BBB permeability during cerebral ischemia-reperfusion injury. Thus we mainly focus on the roles of cav-1.

5.1. Regulation of BBB permeability

Cav-1 is likely to be a critical determinant of the BBB permeability. We are the first to report that the expression of cav-1 was down-regulated in ischemic brains. Furthermore, iNOS- and nNOS- mediated NO production down-regulated the expression of cav-1 in cerebral ischemia-reperfusion injury (77). Cav-1 knockout mice exhibited greater extent of ischemic injury in a model of hindlimb ischemia (134). Cav-1 null mice suffered from microvascular hyperpermeability evidenced by transfer of radio-iodinated bovine serum albumin from the circulation (135). The hyper-permeability was restored by treatment with L-NAME in cav-1 null mice, indicating that the regulation role of cav-1 on BBB permeability is through NO-mediated pathway (135). Cav-1 knockout mice also had higher rates of apoptotic cell death and larger infarction volumes than wild type mice in an experimental ischemic stroke model (7). Furthermore, hydrogen peroxide induced phosphorylation of endothelial barrier disruption was rescued by cav-1 deletion indicating that phosphorylated caveolin-1 Tyr14 plays a crucial role in the regulation of pulmonary microvascular permeability (136). Knockdown of cav-1 in the mouse lung endothelium also selectively decreased the expression of cav-1 by nearly 90% resulting in decreased lung vascular permeability (137). In addition, treatment of a cell permeable peptide of caveolin scaffold domain (AP-CSD) remarkably inhibited eNOS mediated microvascular hyper-permeability (138). Thus, cav-1 plays a pivotal role in regulating BBB permeability during cerebral ischemia-reperfusion injury.

5.2. Impacts on MMPs activation and secretion

Cav-1 protects the BBB from disruption mainly by inhibition of MMPs activity and prevention of the TJs degradation. Chow *et al* reported the colocalization of MMP-2 and caveolin-1 in endothelial cells (8). MMP-2 and its activator MT-MMP were colocalized with cav-1 on the surface of endothelial cells (139). Meanwhile, the synthesized CSD inhibited MMP-2 activity in a dose dependent manner (with K_i = 668nM) (139). Moreover, cav-1 KO mice showed an significant higher MMP-2 activity in heart extraction than in wild type mice (139). Similarly, the overexpression and knockdown of cav-1 in pancreatic carcinoma cells decreased and increased the expression and secretion of MMP-2 and 9 respectively, consequently affecting the ability of tumor cell invasion (140). Besides, enhanced tumor genesis and metastasis in lung cancer model of cav-1 null mice was associated with markedly lower MMP-2/9 secretion and gelatinolytic activity (141). Cav-1 has already been widely accepted as the key regulator of multiple tumor cells progress and invasion through modulation of various MMPs, TIMPs and MT-MMPs (142-144). Although some results were obtained from cancer cells, there are potential meanings for understanding the functions of cav-1 in regulating MMPs in cerebral ischemia-reperfusion injury.

5.3. Effects on tight junction proteins

Cav-1 appears to play a role in the regulation of tight junction proteins. Both occludin and claudin-1 immunoprecipitated cav-1 in MDCK cells (145) and occludin was colocalized with cav-1 in Caco-2 cells (146). The roles of cav-1 on tight junction proteins are still controversial in literature. On one hand, in some studies, cav-1 protects tight junction proteins from the degradation via the down-regulation of MMPs. In primary cultured BMECs, cav-1 was revealed to modulate chemokine-induced BBB permeability by regulating TJ-associated proteins (ZO-1) and AJ-associated proteins (VE-cadherin and ß-catenin) (147). Knockdown of cav-1 with Ad-siCav-1 in BMECs resulted in the loss and the redistribution of TJ- and AJ-associated proteins and in the increase in permeability and migration of monocytic cells across the BMECs. Cavtratin, a synthesized peptide encoding cav-1 scaffolding domain, reversed the effects of Ad-siCav-1 on TJ- and AJ-associated proteins and its permeability to monocytes (147).

On the other hand, other studies suggest that cav-1 decreases TJ protein stability. Cav-1 expression was increased in the vascular endothelium of the site of lesion, and was preceded by the reduction of occludin and claudin-5 at the same sites; this suggests that caveolin-1 might negatively regulate the TJ proteins (148). However, the lesion model they used in the study is rat cortical cold injury. Furthermore, they did not provide evidence to support the negative regulatory effect of caveolin-1 on TJ proteins. Another example showed that cav-1 knockdown attenuated the reduction of ZO-1 in BMECs using an HIV Tat nduced TJ breakdown model (149). A recent study also showed cav-1 mediated internalization of claudin-5 and occludin in a model of CCL2-induced TJ disassembly in brain endothelial cells (150). The clues in the cortical cold injury model, *in vitro* HIV infection model and CCL2-induced TJ disassembly appear to imply that cav-1 may negatively regulate TJ proteins. However, studies on cav-1 null mice showed microvascular hyper-permeability and larger infarction volume when compared to wild-type mice after cerebral ischemia-reperfusion injury (135). Direct evidence is needed to clarify the roles of cav-1 in protecting TJs proteins and BBB integrity in cerebral ischemia-reperfusion injury.

6. INTERACTION BETWEEN CAVEOLIN-1 AND RNS

There is a close interaction between reactive nitrogen species and cav-1. Cav-1 can physically bind to all of NOS isoforms and inhibit NO production. Conversely, NO and peroxynitrite can regulate cav-1 directly or by other means. RNS and caveolin-1 form a feedback loop contributing to BBB insult in cerebral ischemia.

6.1. Cav-1 regulates NO production through binding with NOS

Cav-1 can regulate the expression of NOS via interaction with the caveolin-binding motif. Cav-1 was immunoprecipitated with eNOS in cardiac endothelial cells (151) and lung microvascular endothelial cells (152). Overexpression of caveolin markedly decreased eNOS activity; this inactivation could be blocked by calcium/calmodulin mediated pathway (153, 154). The direct binding of cav-1 and NOS was confirmed by GST-pull down with purified proteins. The binding sequence of cav-1 was located in 82-101 (155). The domain of eNOS that binds to cav-1 was mapped and site-directed mutation of predicted caveolin binding motif was identified (10). Caveolin-1 binds iNOS *in vitro* in a manner similar to that of eNOS and nNOS (10, 156). The interaction of cav-1 and iNOS was reported in various experimental conditions such as inflammation (157) and colon carcinoma (156). The inactivation of nNOS induced by cav-1 was confirmed and the binding motif was identified by the analysis with site mutation (158). The peptides derived from the CSD of cav-1 and -3, but not cav-2, can inactivate eNOS, iNOS, and nNOS (10). The caveolin-NOS interaction appears to be an important signal pathway in various pathological conditions including cirrhosis (159), heart failure(160), inflammation (161), colon carcinoma (157), and atherosclerosis (162).

6.2. NO modulates caveolin-1 levels

NO itself can alter the distribution and abundance of caveolin-1 at the caveolar structures(95). NO donor spermine-NONOate reduced the expression of cav-1 and altered its cellular distribution in the endothelial cells. It also impacted capillary formation at the endothelial cell/tumor cell interface (95). Transcriptional induction of iNOS with inflammatory factors in muscle cells resulted in the decline of cav-1, 2 and 3 levels (163). Similarly, C2C12 cells treated with an NO donor down-regulated the level of caveolin expression; this reduction was not observed in muscle cells obtained from iNOS null mice treated with inflammatory factors (162). In our previous study, treatment of NO donor SNAP decreased the expression of cav-1 protein in both non-ischemic and ischemic brains whereas administration of NOS inhibitors attenuated the down-regulation of cav-1, indicating that NO can down-regulate the expression of cav-1 in ischemic brain injury (9). However, we got somewhat opposite results in human SK-N-MC neuroblastoma cells. NO donor DETA/NO up-regulated caveolin-1 level while the enhancement of cav-1 in the hypoxic human SK-N-MC neuroblastoma cells was attenuated by treatment with L-NAME and iNOS inhibitor 1400W. The increased caveolin-1 level makes the cells have better adaptation in hypoxic environment. The mechanisms of cav-1 in protecting SK-N-MC neuroblastoma cells from oxidative injury are associated with inhibiting iNOS-induced NO production (164). Furthermore, the positive regulation of cav-1 by NO was also observed in human lung carcinoma cell line H460. Nitration of cysteine residue of caveolin-1 resulted in the inhibition of ubiquitination and ubiquitin-proteasome mediated degradation, consequently inducing anoikis by cell detachment (165). The controversial results regarding the regulation of cav-1 by nitric oxide may be due to the different cell types and experimental conditions. It is necessary to note that the positive regulations of cav-1 by NO were found in tumor cells. So, it is likely that for some reason, tumor cells adopt different regulatory ways.

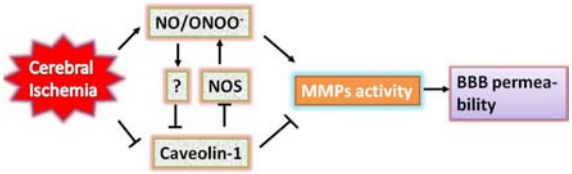

Figure 2. Proposed mechanisms of the inter-regulation between caveolin-1 and nitric oxide induced MMPs activation and BBB breakdown. Cerebral ischemia-reperfusion induces NO production and decreases caveolin-1 level. On one hand, caveolin-1 directly binds to nitric oxide synthase (NOS) and decreases production of NO; on the other hand, NO negatively regulates caveolin-1 expression. The NO produced and decreased level of caveolin-1 further activates MMP-2 and MMP-9, disrupting tight junction proteins and matrix, finally inducing BBB hyperpermeability.

In summary, on one side, nitric oxide can inhibit the expression of caveolin-1; on the other side, the inhibition of caveolin-1 increases NOS activity and produces more NO. As summarized in Figure 2, the positive feedback reinforces the effects of both caveolin-1 and nitric oxide on BBB insult during cerebral ischemia and reperfusion injury.

7. SUMMARY AND PERSPECTIVE

Stroke is the secondary cause of death and the leading cause of disability in human diseases worldwide. BBB damage is a critical pathophysiological process in stroke, contributing to the formation of brain edema and infarction enlargement. There are very limited therapeutic approaches available for manipulation of the BBB disruption clinically. Understanding the molecular mechanisms of the BBB breakdown in cerebral ischemia-reperfusion injury will greatly impact current clinical management and prognosis of stroke. Protecting the BBB from breakdown and avoiding resulting edema formation are the most important research directions in the field of cerebral ischemia-reperfusion injury. The rapid growth of the studies on these aspects has generated more than 4000 publications during past several decades. The molecules that cause BBB opening are extremely complicated and can be generally divided into three categories: free radicals that directly modify membrane lipids and other regulatory proteins, proteases which cleave the organized sealing proteins and matrix around them, and other regulatory proteins that take effect upon ischemia-induced hypoxia and neuroglycopenia. Recent progress indicates that the interactions of free radicals, MMPs and caveolin-1 play pivotal roles in the disruption of the BBB integrity in ischemic stroke. Drug discoveries targeting the interactions of free radicals, MMPs and caveolin-1 may lead to the development of novel therapeutic strategies for manipulating of the progress of BBB disruption and subsequent neurological dysfunction in stroke treatment.

Nevertheless, due to the short lifetime of many free radicals, current techniques that can be used to directly trace the production are very limited. Thus, there is no direct and unambiguous evidence to clarify the roles of free radicals and caveolin-1 in regulating MMPs activity, TJs degradation and BBB permeability during cerebral ischemia-reperfusion injury. As a structural and scaffold protein of caveolae, caveolin-1 can negatively regulate the proteins and molecules in a variety of signal transduction pathways and cellular functions. The protective effects of caveolin-1 in the BBB integrity could be potentially explained by the indirect interactions with other cellular signal molecules. As a scaffold protein, caveolin-1 binds and inhibits many proteins that contain the CAV-binding site. MMP family members (MMP-2, 3, 9) containing CAV-binding site are considered as potential binding targets. Although the inactivation of MMP-2 by caveolin-1 scaffold domain was previously reported (8), there is no evidence indicating that caveolin-1 directly binds to MMPs.

More importantly, the impacts of the interactions of free radicals, MMPs and cav-1 on many important aspects in stroke have yet to be addressed. For example, primary intracerebral hemorrhage (ICH) is the most devastating form of stroke which accounts for 10~15% of stroke victims and results in more severe neurological deficits and higher mortality rates than ischemic stroke. It will be interesting to address how the interaction of free radicals, MMPs and caveolin-1 impacts the pathological process of hemorrhagic stroke.

In conclusion, further studies on the interactions of free radicals, MMPs and cav-1 in the BBB permeability may offer an opportunity to develop novel therapeutic strategies for stroke treatment.

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Abbreviations: ROS: reactive oxygen species, RNS: reactive nitrogen species, BBB: blood-brain barrier (BBB), MMPs: matrix metalloproteinases, TJs: tight junctions, ECM: extracellular matrix, NO: nitric oxide, PDCs: peroxynitrite decomposition catalysts, I/R: ischemia/reperfusion, NOS: nitric oxide synthase, Cav-1: caveolin-1, MMPs: matrix metalloproteinases, CNS: central nervous system, AJs: adherent junctions, JAM-1: junctional adhesion molecule-1, ZOs: zonula occludens, MT-MMPs: membrane-bound MMPs, TIMP: tissue inhibitor of metalloprotease, SOD: superoxide dismutase, TEER: transendothelial electric resistance, BMECs: brain microvascular endothelial cells, nNOS: neuronal NOS, iNOS: inducible NOS, eNOS: endothelial NOS, L-NAME: Nώ-nitro-L-arginine, SIN-1: 3-morpholinosydnonimine, CSD: caveolin scaffold domain, ICH: intracerebral hemorrhage, NADPH: nicotinamide adenine dinucleotide phosphate

Key Words: Blood-brain barrier, Free radicals, Nitric oxide, Caveolin-1, Cerebral ischemia, Reactive oxygen species, Reactive nitrogen species, Review

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