Occludin regulation of blood–brain barrier and potential therapeutic target in ischemic stroke

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Abstract:

Occludin is a key structural component of the blood–brain barrier (BBB) that has recently become an important focus of research in BBB damages. Many studies have demonstrated that occludin could regulate the integrity and permeability of the BBB. The function of BBB depends on the level of occludin protein expression in brain endothelial cells. Moreover, occludin may serve as a potential biomarker for hemorrhage transformation after acute ischemic stroke. In this review, we summarize the role of occludin in BBB integrity and the regulatory mechanisms of occludin in the permeability of BBB after ischemic stroke. Multiple factors have been found to regulate occludin protein functions in maintaining BBB permeability, such as Matrix metalloproteinas-mediated cleavage, phosphorylation, ubiquitination, and related inflammatory factors. In addition, various signaling pathways participate in regulating the occludin expression, including nuclear factor-kappa B, mitogen-activated protein kinase, protein kinase c, RhoK, and ERK1/2. Emerging therapeutic interventions for ischemic stroke targeting occludin are described, including normobaric hyperoxia, Chinese medicine, chemical drugs, genes, steroid hormones, small molecular peptides, and other therapies. Since occludin has been shown to play a critical role in regulating BBB integrity, further preclinical studies will help evaluate and validate occludin as a viable therapeutic target for ischemic stroke.

Keywords:

Blood-brain barrier, ischemic stroke, occludin

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Introduction

Ischemic stroke is characterized by the occlusion of the cerebral artery or arteries supplying the brain tissues, resulting in neuronal death within minutes in the corresponding brain regions.[1,2] Accumulating evidence support that blood-brain barrier (BBB) damage is an early event after ischemic stroke, leading to pathophysiological damages in the brain. BBB damage further results in progressive neuronal death and cerebral edema, even intracerebral hemorrhage transformation after ischemic stroke.[3] Therefore, preserving BBB integrity is of critical importance in designing treatments for ischemic stroke.

Occludin, as a member of tight junction proteins, is a key structural component of the BBB,[4] and occludin degradation can be seen in the course of ischemic stroke, leading to the disruption of BBB.[5-8] In this review, we summarize the role of occludin in maintaining BBB integrity and the molecular mechanisms of occludin interruption, leading to alteration of the BBB permeability after ischemic stroke.

Blood–Brain Barrier and Tight Junctions

Many previous studies demonstrated that a physical barrier, which was subsequently identified as BBB, exists between the central nervous system and the peripheral circulation to prevent the toxic and harmful substances from invading brain tissue.[9,10]

BBB is mainly composed of brain endothelial cells, tight junctions, astrocyte end-feet, pericytes, microglial cells around blood vessels, and basement membrane.[11] BBB prevents the brain from neurotoxins, neurotransmitters, and macromolecules, and passively transporting water-soluble nutrients or metabolites and gases, maintaining the balance of the microenvironment in the brain.[12]

Compared to epithelial tight junctions, endothelial tight junctions in BBB are highly special in molecular structure with more restrictive paracellular diffusion barrier, lower transcytotic vesicles and more sensitive to the microenvironment.[13] Tight junctions between endothelial cells are made up of zonula occludens (ZO) and transmembrane proteins, such as occludin, claudins, junctional adhesion molecules, and tricellulin, linked to the cytoskeleton and cytoplasmic scaffold proteins.[14] The changes of the sealing proteins in conformation or modified adjusting have a direct effect on the state of tight junctions and further, affect the permeability of BBB.[15]

Recently, occludin has been found to play a critical role in BBB integrity. Many studies showed that occludin degradation or abnormal occludin could be found to increase BBB permeability in various central nervous system diseases,[16] especially in ischemic stroke, disturbing the stability and normal function of the brain. Therefore, it is essential to further understand the function and regulation mechanism of occludin.

The Structure of Occludin and Blood-Brain Barrier

Furuse *et al.* originally extracted the proteins from poultry tissues and identified it as an integral membrane protein in epithelial and endothelial cells.[17] Occludin is indispensable for barrier integrity in diverse endothelial cell models,[18] but some studies reported that other tight junctions in epithelial cells were able to maintain morphological integrity in occludin deficient models,[19] suggesting that occludin is prone to protecting barrier function rather than assembly in BBB.

The barrier function of occludin depends on its special structure domains. Occludin is a 65 kDa integral membrane protein with 502 amino-acid residues, which includes two extracellular loops, one intracellular loop, and its carboxyl and amino-terminals oriented toward the cytoplasm. The first extracellular loop (ECL1) of occludin has a very high content of tyrosine and glycine residues (about 55aa). The tyrosine residues are involved in forming hydrophobic interactions and H-bonds, while glycine residues provide flexibility. The second extracellular loop (ECL2, about 45aa) is rich in tyrosine residues and contains two cysteines to form disulfide

bridges in the oxidizing environment, which is sensitive to hypoxia and occurs homo-oligomerization.[20] Compared to ECL1, ECL2 serves as the main binding domain, which interacts with other tight junctions and regulates the function of other tight junctions. In addition, C-terminal cytoplasmic of occludin (about 259aa) is rich in serine, threonine, and tyrosine residues and directly connects to ZO-1 and actin cytoskeleton.[21] It contains the main binding sites for regulatory molecules, such as connexin 26 or different kinases.[22] Both external loops as well as the transmembrane and the C-terminal cytoplasmic domains of occludin, are important for the regulation of paracellular permeability between adjacent cells.

The Regulation of Occludin and Blood–Brain Barrier Integrity

At present, multiple factors have been found to regulate occludin functions on BBB permeability,[23] such as matrix metalloproteinases (MMPs)-dependent degradation, phosphorylation, ubiquitination, and other cytokines.

Matrix metalloproteinase-dependent occludin degradation

MMPs are secreted as zymogens and cleaved to be active. *In vivo* and *in vitro* evidence show that the levels of active MMP-2 and MMP-9, which are extremely low in normal brain tissue, mediate occludin degradation in pathological conditions.

BBB was damaged via vascular endothelial growth factor (VEGF)-mediated MMP-9 activation in a hypoxia mouse model, leading to the reduction of occludin expression, but there was no significant change in the expression of claudins and ZO-1.[24] Besides, the activity of MMP-9 could be suppressed by the inhibition of the nuclear factor-kappa B (NF-κB) pathway in the transient middle cerebral artery occlusion (tMCAO) mouse model, and the expression of occludin, JAM-A and ZO-1 proteins in brain tissue was elevated, which helps to protect BBB integrity.[25]

In addition, the expression of occludin significantly decreased by the MMP-2/MMP-9 activation in brain microvascular endothelial cells (BMECs) in an OGD/R-injury neurovascular unit model, which involved the mitogen-activated protein kinase (MAPK) pathways.[26] Accumulating evidence shows that activation of MAPK signaling pathways contributes to BBB damage,[27] leading to MMP-9 expression increase and reducing the level of occludin, ZO-1, and claudin-5 in BMECs.

Occludin Phosphorylation
Occludin phosphorylation has been identified as an important regulatory mechanism in regulating BBB integrity. Numerous studies showed that the

BBB permeability is related to the status of occludin phosphorylation at serine/threonine or tyrosine.[28-31] Occludin has multiple phosphorylation sites, among which the Ser-507, Thr-382, Ser-490, and Ser-338 are classical phosphorylation sites. The state of occludin phosphorylation has different effects on BBB permeability, depending on the phosphorylation types of occludin or diverse signaling pathways.

Some studies have described the regulation of occludin by protein kinase c (PKC). An *in vitro* study in LLC-PK1 cells showed that activation of PKC with 12-O-tetradecanoylophorbol-13-acetate (tissue plasminogen activator [TPA], a PKC activator) reduced threonine phosphorylation of occludin protein, resulting in increased paracellular permeability.[32] However, TPA improved barrier function and upregulated the expression of occludin protein via activation of PKC.[33] Further, inhibition or knockdown of PKC induced dephosphorylation of occludin on threonine residues (T403 and T404) in Caco-2 and MDCK cell monolayers.[34] These studies indicated that PKC may have opposite effects on occludin protein regulation in the signaling pathway under different conditions.[35]

In addition, VEGF-induced occludin phosphorylation increased BBB permeability,[36] while inhibition of VEGF-induced PKC-β activation reduced the barrier permeability by preventing occludin phosphorylation at Ser-490.[37] Moreover, an *in vivo* study reported that Ser-490 was a special key site for regulating the interaction of occludin with other tight junction binding partners (e. g. ZO-1) in VEGF-treated retinal endothelial cells.[38]

Further, Walsh *et al.* reported that vascular endothelial cadherin could transmit physiological shear signals to occludin via the Tiam1/Rac1 signaling pathway in brain microvascular endothelial cells (BMECs) model, resulting in Tyr-occludin dephosphorylation and reduced BMECs permeability.[39] Moreover, another study demonstrated that cyclic strain (5% strain, 60 cycles/min, 24 h) induced the expression of occludin/ZO-1 proteins upregulation in endothelial cells, with the tyrosine phosphorylation of occludin reduced and the serine/threonine phosphorylation of ZO-1 increased.[40] Besides, there are many neurotransmitters involved in tyrosine phosphorylation of occludin. Glutamate increased tyrosine phosphorylation and decreased threonine phosphorylation of occludin in brain microvascular endothelial cells through N-methyl-D-aspartate or alpha-amino-3-hydroxy-5-methylisoxazole-4-propionate/kainite receptors, resulting in disruption of the BBB.[41] Treatment of microvascular endothelial cells with protein phosphatase type 2A (PP2A) showed that PP2A dephosphorylated

the tyrosine residue of occludin, leading to the reduction of occludin expression. Moreover, inhibiting the activity of PP2A maintained the BBB integrity via hyperphosphorylation of tyrosine residues of occluding.[42] When monolayer microvascular endothelial cells were exposed to septic insults, PP2A reduced serine and threonine phosphorylation of occludin, leading to increased monolayer permeability.

Occludin ubiquitination
Ubiquitin is a heat-stable 76-amino acid protein, which was found in eukaryotic cells and coupled to lysine residues in an ATP-dependent manner as a monomer or polymer, guiding the protein to the proteasome degradation pathway.[43] Ubiquitination is an important mechanism for regulating the function of target proteins by modifying their intracellular transport and degradation within endothelial tight junctions.

Lui and Lee *et al.* reported that occludin degradation was associated with Itch and UBC-4 (an ubiquitin-conjugating enzyme), which was mediated by dibutyryl-cAMP pathway, resulting in occludin ubiquitination to disrupt tight junctions in blood and testosterone barrier cells.[44] Furthermore, Nedd4-2, a member of E3 ubiquitin ligase, was co-immunoprecipitated with occludin through interacting on the conserved PY motif in the C-terminal of occludin, suggesting that Nedd4-2 ubiquitinated occludin and induced occludin degradation.[45] Moreover, silencing Nedd4-2 upregulated the expression of occludin and reduced paracellular permeability in mpk-CCDc14 cells. Recently, Murakami *et al.* demonstrated that occludin ubiquitination regulates endothelial permeability in bovine retinal endothelial cell models via the VEGF-mediated pathway.[46] It was shown that phosphorylation of occludin at Ser-490 is necessary for its ubiquitination after VEGF treatment. Finally, occludin ubiquitination induces the endocytosis of related tight junction proteins (such as claudin-5 and ZO-1), which ultimately lead to the destruction of the BBB.[47]

The interaction with cytokines
Cytokines are involved in a multitude of molecular mechanisms in regulating occludin expression and mediating changes in BBB integrity.

Tumor necrosis factor-α
Tumor necrosis factor-α (TNF-α) is known to induce changes in endothelial cell morphology and permeability, but the mechanisms have not been extensively characterized.[48] Ni *et al.* demonstrated that TNF-α induced upward-band shift of occludin (phosphorylation) in the human cerebral endothelial cell line (hCMEC/D3) by transient stimulation of p38MAPK and ERK1/2 pathway, increasing cerebral endothelial cells permeability and

leading to BBB disruption.[49] In renal endothelial cells, TNF-α-induced barrier dysfunction or the reduction of occludin expression is crucially dependent on the Rho/MLCK signaling pathway.[50] TNF-α also downregulated occludin expression by activating HIF-1α/VEGF/VEGFR-2/ERK signaling pathway, which was inhibited by propofol.[51] In addition, platelet endothelial cell adhesion molecule-1 (PECAM-1) expression is involved in maintaining barrier function in endothelial cells. Combined treatment of TNF-α and dengue virus caused decreased barrier function, altered distribution of PECAM-1, and lower level of occludin protein in human endothelial cells.[52] Furthermore, NADPH in human astrocytes enhanced TNF-α-induced barrier dysfunction, accompanying with the activation of MMPs and reduction in occludin expression.[53] Moreover, decreasing NADPH activation by inhibiting TNF-α improves the barrier function through upregulating the expression of occludin proteins.[54] These studies suggest that TNF-α degrades occludin and promotes BBB damage by multiple signaling pathways.

Interleukin-1β

As one of the pro-inflammatory cytokines, interleukin-1β (IL-1β) plays an important role in regulating the expression of occludin in inflammation.[55] Toshiaki Abe *et al.* found in cultured human retinal pigment epithelial cell line (ARPE-19) that IL-1β downregulated the expression of occludin but upregulated claudin-1, compared with the control medium,[56] suggesting that IL-1β induces the loss of occludin proteins. Some studies explored the mechanisms of interaction between IL-1β and occludin. The activation of ATP/P2 X 7R (a unique purinergic receptor) signaling pathway induced degradation of occludin, and ZO-1 proteins were associated with the release of IL-1β and enhancement of the MMP-9 activity in the human model of BBB *in vitro*.[57] Besides, the NF-κB pathway is essential for IL-1β to induce the redistribution of occludin and ZO-1 proteins, resulting in BBB disruption in cultured human corneal epithelial cells.[58] This was consistent with a recent study that Mdivi-1 (a selective dynamin-related protein-1 inhibitor) alleviated the brain edema after subarachnoid hemorrhage to inhibit NF-κB dependent inflammation via suppressing occludin degradation and IL-1β release.[59] Further, IL-1β-induced MMP-9 expression and activation in pericytes suppressed the expression of occludin proteins in the BBB model and led to increased BBB permeability, which was regulated by the NOTCH3/NF-κB signaling pathway.[60] Inhibiting IL-1β and montelukast (leukotriene receptor type 1 antagonist) enhanced the expression of occludin and ZO-1 proteins and protected against BBB disruption.[61,62] However, another study reported that IL-1β increased the expression of claudin-1, but there was no significant change in the expression of occludin in cultured HaCaT keratinocytes. The different effects may be associated with the stage of skin healing, but the underlying mechanism is still not clear.[63]

Interferon-γ

Interferon (IFN-γ), secreted from activated T and natural killer cells, not only plays immunomodulatory roles in inflammation but also modifies endothelial barrier function.[64] Studies showed that IFN-γ maintains BBB integrity by enhancing the expression of occludin and ZO-1 in the experimental autoimmune encephalomyelitis mice model.[65] However, other studies reported that IFN-γ has the opposite effect on BBB integrity.[66] For example, occludin expression significantly decreased after IFN-γ treatment in human umbilical vein endothelial cell layers,[67] while occludin protein disassembly was observed when exposed to IFN-γ in BBB transwell model *in vitro*.[68] In addition, the study demonstrated that the extent of BBB damage was related to the concentration of cytokines such as IFN-γ in a dose-dependent manner via JNK signaling pathways.[69] In summary, these studies showed that IFN-γ regulates BBB function through interacting with occludin in diverse experimental models.

Hepatocyte growth factor

Hepatocyte growth factor (HGF) is a multifunctional cytokine, including mitogenic, motogenic, morphogenic, angiogenic, and anti-apoptotic activities in diverse types of cells,[70] which help alleviate ischemia-induced injuries via anti-apoptotic and angiogenic activities.[71,72] One study demonstrated that human recombinant HGF relieved the extent of BBB disruption in a microsphere-induced cerebral embolism rat model, and mitigated the reduction of occludin expression.[73] However, there was an opposite finding that treatment of human microvascular endothelial cells with HGF decreased transendothelial cell resistance as well as occludin expression, and increased paracellular permeability.[74] In general, HGF plays its role in regulating the function of tight junctions by altering the phosphorylation state of occludin and it does not change the phosphorylation of ZO-1.[75] These studies indicate that HGF can regulate the expression level of occludin, leading to changes in the function of tight junctions, but the mechanism of HGF in regulating phosphorylation of occludin is still unknown. Further investigations are needed to clarify the mechanism.

Occludin and Ischemic Stroke

Ischemic stroke is one of the most common diseases with high mortality and disability, which accounts for around three-fourths of all strokes.[76] Many studies have demonstrated that disruption of BBB is an early event after ischemic stroke, which may develop into

hemorrhagic transformation at later time points following ischemia-reperfusion.[77-79] Occludin serves as one of the key structural tight junction proteins for BBB integrity. Animal and human studies have indicated that occludin degradation is frequently seen in ischemic stroke and contributes to BBB injury.[5-8] Therefore, we focus our attention here on occludin degradation and regulation in ischemic stroke.

Occludin degradation into fragments by matrix metalloproteinases in ischemic stroke

Our previous studies demonstrated that occludin degradation and claudin-5 redistribution is seen in isolated ischemic cerebral microvessels from MCAO rat models, causing the disruption of BBB integrity.[7] In other studies, increased MMP-2/9 activation in the ischemic brain contributes to BBB disruption through enhanced occludin degradation.[5-6,80] Further, Pan *et al*. demonstrated that the level of blood occludin fragments increases proportionately to the extent of BBB damage at the early stage of cerebral ischemia in an MCAO rat model,[81] and most importantly, the worst BBB damage occurs at 4.5-h after stroke onset, coincident with the peak level of occludin fragments in the peripheral blood. These studies indicate that blood occludin may be a biomarker of BBB damage, thus could serve as a potential predictor of hemorrhagic transformation in ischemic stroke patients.[82] The level of serum occludin is detected by enzyme-linked immunosorbent assay at present. However, this method could not distinguish the fragments of occludin from the full-length proteins in the serum. Further studies are required for developing specific method to improve sensitivity and specificity.

Occludin regulation in ischemic stroke

Owing to BBB damages playing a pivotal role in ischemic stroke, there are many studies on occludin regulation to protect BBB in cerebral ischemic animals or patients.

Normobaric hyperoxia treatment

As tissue hypoxia is a critical event in the pathophysiology of ischemic stroke, supplement of oxygen to ischemic tissue has long been thought of as a logical stroke treatment strategy.[83] Treatment with normobaric hyperoxia (NBO, 100% oxygen) increases ischemic tissue oxygenation by maintaining the penumbral PO_2 level close to the pre-ischemic level.[84] Early NBO treatment is neuroprotective via delaying the progression of ischemic brain tissue necrosis, which is equivalent to saving time and expanding the window of opportunity for reperfusion therapies.[85,86] Importantly, combination treatment of NBO and recombinant tPA in the MCAO rat model could lessen BBB disruption and reduce hemorrhagic transformation, compared with rats which underwent delayed tPA treatment at 5 or 7 h postischemia.[87] These studies indicate that NBO

treatment after ischemia stroke onset helps to rescue the ischemic penumbra and microvessels, and has a great potential for serving as a promising adjuvant therapy to extend time window of tPA thrombolysis or thrombectomy for ischemic stroke.[88]

Our previous studies also investigated the mechanisms of NBO on BBB protection. NBO treatment played a vital role in slowing the progression of BBB disruption via inhibiting the activity of MMPs and the consequent occludin degradation.[5] In addition, the study of Liu *et al*. suggested that NBO treatment protects the BBB against ischemic damages by reducing MMP-9 activity and enhancing the expression level of occludin proteins in microvessels, which was inhibited by gp91[phox] (also called Nox2) in an MCAO mouse model.[89] In addition to combined treatment with NBO and tPA, there was another report that NBO plus minocycline could effectively reduce the extent of ischemic brain injury and protect BBB due to inhibition of MMP-2/9-mediated occludin degradation and alleviation of caspase-dependent and independent apoptotic pathways.[90] Further, Shi *et al*. demonstrated that NBO could reduce the level of blood occludin in acute ischemic stroke patients, alleviate BBB permeability, and improve outcome of stroke patients with tPA thrombolysis.[91] These results indicate that NBO is very effective in reducing occludin degradation through inhibition of MMP-2/9 activity or gp91[phox] (also called Nox2) in ischemic brain tissue and alleviating the extent of BBB damage in ischemic stroke, which makes NBO a promising auxiliary approach to expand the narrow time window of reperfusion therapies for ischemic stroke.[92] Our previous study has shown that NBO can be applied in ischemia/reperfusion injury patients, especially for tPA thrombolysis in ischemic stroke in the clinic.

Chinese medicines

A lot of traditional Chinese medicines have been reported to reduce the ischemic brain damages by regulating BBB permeability with various therapeutic mechanisms. In the oxygen-glucose deprivation/recovery (OGD/R)-injured neurovascular unit model, Zhao *et al*. demonstrated that cryptotanshinone inhibited MAPK signaling pathway to ameliorate neuron apoptosis and elevated the expression of occludin protein through down-regulation of MMP-9 expression.[26] Liu *et al*. demonstrated that green tea polyphenols promoted mRNA or protein expression of occludin after ischemia in the MCAO rat model by inhibiting PKCα activity to reduce BBB leakage.[93] The hairy root extract of Angelica gigas was proved to increase the expression of occludin in MCAO rats through activation of the PI3K/Akt pathway, leading to the alleviation of BBB disruption.[94] Considering the positive outcomes, it is likely that multiple signaling pathways are involved in Chinese medicines mediated

occludin regulation in ischemic stroke, including the MAPK signaling pathway, PKCα activity, and PI3K/AKT signaling pathway. The effective and proven Chinese medicines could be considered as a therapeutic or preventative strategy for patients who have or are at high risk of suffering a stroke.

Repurposing of conventional chemical drugs

Recently, multitudes of evidence provide a new viewpoint for treating cerebral ischemic with conventional chemical drugs. For example, in addition to antispermatogenic function, adjudin had been shown that its anti-neuroinflammation effect plays an important role in preventing cerebral reperfusion injury in the tMCAO mouse model by suppressing the NF-κB pathway, inhibiting the elevated MMP-9 activity and increasing protein expression of occludin.[25] Moreover, the combination of NBO and minocycline (a broad-spectrum tetracycline antibiotics) significantly inhibited MMP-9 activity and occludin degradation in the rat MCAO model.[90] Furthermore, thalidomide, an old drug with anti-inflammatory and anti-cancer properties, downregulated the expression of TNF, IL-1 β, and MMP-9 that preserves occludin and attenuates BBB disruption.[95] These results show that certain conventional chemical drugs can be repurposed for preserving BBB integrity through inhibiting the NF-κB pathway, TNF, IL-1 β, or MMP-9 activity to increase occludin. However, further clinical studies are required to validate the effectiveness of these old drugs in treating stroke patients.

Small molecular peptides

Cystatin C, serves as a biomarker of renal function injury, widely exists in brain tissue, and the level of its expression increases in stroke. Yang *et al.* demonstrated that overexpression of cystatin C possesses neuroprotective effect on maintaining BBB integrity by increasing the expression of caveolin-1 and occludin after ischemic brain injury.[96] Although the treatment of cystatin C has rescued occludin by increasing caveolin-1 expression, the specific mechanism by which cystatin C regulates occludin will require additional investigation. N-acetylcysteine (NAC), a precursor of glutathione, contains free sulfhydryl of disulfide bonds and promotes synthesis of GSH. Due to its anti-oxidant and anti-inflammatory property, NAC is broadly applied to cardiovascular disease at present.[97] Wang *et al.* demonstrated that NAC in MCAO diabetic mice reduced occludin glycation and alleviated BBB permeability, showing cerebral protection by correcting the ratio of methylglyoxal/glutathione.[98] As an antioxidant, NAC offers a preventative approach to protect against the worsened stroke outcome in diabetics through reducing occludin glycation. Further clinical studies are still needed.

Steroid hormones

Vitamin D and glucocorticoid, as members of the steroid hormones family, bind to their receptors in the cytoplasm and nucleus, respectively, to trigger the strong biological effect. It was shown that exposure of neuronal cells to hypoxia/reoxyenation triggers a series of cascade reaction, including the increased formation of intracellular reactive oxygen species (ROS), increased activation of NF-κB signaling pathways, leading to augmented expression of MMP-9 that mediated BBB disruption through degradation of occludin, claudin-5, and ZO-1. Vitamin D treatment prevented BBB disruption by inhibiting ROS production and NF-κB activation in a vitamin D receptor-dependent manner.[99] However, another steroid hormone, the glucocorticoid stress hormone (GC), has the opposite effect. GC receptor signaling in endothelial cells can be activated under ischemic conditions, and GC reduced the expression level of occludin through binding to the GC receptor, contributing to worsening the ischemic infarct.[100] The role of steroid hormones acting on occludin has opposite views about regulating occludin expression through different signaling pathways, including inhibition of the NF-κB pathway or activation of the GC receptor. However, the basic study should further explore the specific mechanisms.

Other therapies

In vitro and *in vivo* evidence show that intravenous immunoglobulin could rescue ischemic neuronal cell by reducing leukocyte filtration and blocking BBB permeability, and importantly, it prevented the ischemia-induced downregulation of tight junction protein occludin and claudin-5.[101] This study demonstrated that immunoglobulin indeed can protect BBB by upregulating occludin, but the mechanisms are not clear. A recent study indicates that inhibition of miR-210 with its complementary locked nucleic acid oligonucleotides (miR-210-LNA)-mediated neuroprotection via preserving the expression of junction protein occludin in neonatal hypoxic-ischemic brain injury.[102] This result is limited to the role of miR-210 in a hypoxic-ischemic model. It is not clear whether miR-210-LNA treatment is effective in ischemic animal models, such as MCAO. Moreover, in an MCAO/R mice model, VEGF treatment aggravated BBB disruption by increasing LOC102640519 and HOXC13 through inhibition of ZO-1, occludin, and claudin-5,[103] which provides another therapeutic strategy for VEGF-based treatment for stroke patients.

Ischemic preconditioning (IP) has been shown to induce changes in tight junctions to protect against BBB breakdown after MCAO through activation ERK1/2.[104] In addition, the sphingosine kinase-2 contributes to protecting BBB integrity in hypoxic

preconditioning-treated animals via generating S1P that participates in the maintenance of occludin at cytoskeletally linked cell junctions.[105] However, there have not enough clinical studies on IP alleviating BBB damage in ischemic stroke at present, and the role of IP in regulating occludin in clinic study needs to be done.

In addition, the emerging nanotechnology helps deliver beneficial drugs across the BBB. Some studies have reported that drugs loaded into the nanotechnology system could permeate through the BMECs.[106,107] The mechanism for this delivery is most likely dependent on LDLs receptor-mediated endocytosis by the BMECs,[108] and may not influence the tight junctions of the brain endothelium.[109] Further studies on possible mechanisms will need to be explored in future.

Conclusions

In summary, occludin is a transmembrane protein of tight junctions that regulates the integrity and permeability of the BBB. Various factors have been found to regulate occludin expression, such as MMPs-dependent degradation, phosphorylation, ubiquitination, and other cytokines. The mechanisms of regulating occludin protein in tight junctions involve many diverse signaling pathways [Table 1], including NF-κB, MAPK, PKC, RhoK, and ERK1/2. Further, the different sites of phosphorylation or biological environments may impact

Table 1: Modification of occludin protein regulating TJs

Experimental model	Effect on occludin	Mechanism	Reference
Hypoxia mice model	Degradation	Hypoxia-induced MMP-9 activation↑	[24]
tMCAO mice model	Expression↑	MMP-9 activity ↓ by inhibition of NF-κB pathway	[25]
OGD/R-injury model	Degradation	MMP-2/9 activity↑ by activation of MAPK pathway	[26]
LLC-PK1 cells	Thr-dephosphorylation	Activation of PKC	[32]
Caco-2 and MDCK cell monolayers	Thr-404,Thr-403 dephosphorylation	Inhibition of PKC	[34]
	Ser-490 Phosphorylation	Activation of PKC-β	[37]
BMECs	Tyr-phosphorylation	Shear signal activation of Tiam1/Racl signal pathway	[39]
BMECs	Tyr-phosphorylation and Thr-dephosphorylation	Activation of NMDA or AMPA/KA receptors	[41]
Microvascular endothelial cells	Tyr-dephosphorylation	Activation of PP2A	[42]
Sertoli cell	Ubiquitination	Dibutyryl-cAMP mediated the level of Itch and UBC4↑	[44]
Mpk-CCDc14 cells	Ubiquitination	Nedd4-2 mediated immunoprecipited	[45]
BRECs	Expression↓	VEGF-induced occludin ubiquitination	[46]
HCMEC/D3 cells	TNF-α induced phosphorylation	Stimulation of p38MAPK and ERK1/2	[49]
Renal endothelial cells	TNF-α induced expression↓	Activation of Rho/MLCK pathway	[50]
HCMEC/D3 cells	TNF-α induced expression↓	Activation of Hif-1a/VEGF /VEGF-2/ERK	[51]
Human endothelial cells	TNF-α induced expression↓	PECM-1 redistribution	[52]
HBMECs and HAs	TNF-α induced expression↓	NADPH oxidase activity↑	[53]
RPE cells	IL-1β induced Expression↓	Unknown	[56]
HCMEC/D3 cells	IL-1β induced expression↓	Activity of MMP-9 ↑ by activation of P2X7R	[57]
HCE cells	IL-1β induced expression↓	Activation of NF-κB pathway	[58]
In vitro BBB model	IL-1β induced expression↓	Activation ofNOTCH/NF-κB pathway	[60]
EAE mice model	IFN-γ induced exipression↑	Unknown	[65]
BALB/c mice model	IFN-γ induced exipression↓	Unknown	[66]
HUVEC layers	IFN-γ induced exipression↓	Unknown	[67]
BBB transwell model	IFN-γ induced diassembled	Unknown	[68]
Cerebral embolism rats model	HGF-induced expression↑	Unknown	[73]
Human vascular endothelial cells	HGF-induced expression↓	Unknown	[74]

MMP: Matrix metalloproteinase, tMCAO: Transient middle cerebral artery occlusion, NF-κB: Nuclear factor κB, OGD/R: Oxygen-glucose deprivation/recovery, MAPK: Mitogen-activated protein kinase, PKC: Protein kinase C, LLC-PK1: The cell junctional complex in the pig kidney, Caco-2: Colorectal carcinoma, MDCK: Madin-Darby Canine Kidney, BREC: Bovine retinal endothelial cells, BMECs: Brain microvascular endothelial cells, NMDA: N-methyl-D-aspartate, AMPA/KA: Alpha-amino-3-hydroxy-5-methylisoxazole-4-propionate/kainate, PP2A: Protein phosphatase type 2A, mpk-CCDc14: Murine cortical collecting duct, HCMEC: Human cerebral microvascular endothelial cell, PECM-1: Platelet endothelial cell adhesion molecule-1, HBMECs: Human brain microvascular endothelial cells, NADPH: Nicotinamide adenine dinucleotide phosphate, ERK: Extracellular signal-regulated kinase, MLCK: Myosin light chain kinase, P2X7R: Ionotropic purinergic receptor, EAE: Experimental autoimmune encephalomyelitis, HUVEC: Human umbilical vein endothelial cell Layers, TJs: Tight junctions, RPE: Rential pigment epithelial, HCE: Human corneal epithelial, BBB: Brain blood barrier, HGF: Hepatocyte growth factor, UBC4: Ubiquitin-conjugating enzyme 4, VEGF: Vascular endothelial growth factor, Hif-1: Hypoxia-inducible facor-1, Tiam1/Raci: Tlymphoma invasion and metastasis inducing factor1/ras-related C3 botulinumtoxin substrate, TNFα: Tumor necrosis factor-α, IL-1β: Interleukin-1β, IFN-γ: Interferon-γ

Table 2: Occludin and ischemic stroke therapy

Treatment or drugs	Experimental model	Mechanism	Occludin effect	Reference
NBO plus tPA	MCAO rats model	Inhibition of MMP-9 activity	Expression↑	[87]
NBO	MCAO rats model	Inhibition of MMP-9 activity	Expression↑	[5]
NBO	MCAO mice model	Inhibition of gp91^{phox} (or called Nox2)	Expression↑	[89]
NBO plus minocycline	MCAO rats model	Inhibition of on MMP-2/9 activity and apoptotic pathways	Expression↑	[90]
NBO	Ischemic stroke patient	Inhibition of MMP-9 activity	Blood level↓	[91]
Cyptotanshinone	OGD/R injured NVU model	Down-regulation of MMP-9 activity	Expression↑	[26]
Greeen tea polyphenols	MCAO rats model	Inhibition of PKCα activity	Expression↑	[93]
Angelica gigas	MCAO rats model	Activation of the PI3K/AKt pathway	Expression↑	[94]
Adjudin	tMCAO mice model	Suppression of the NF-κB	Expression↑	[25]
Minocyline	MCAO rats model	Inhibition of MMP-9 activity	Expression↑	[90]
Thalidomide	MCAO mice model	TNF-α, IL-1β production↓ and MMP-9 activity↓	Expression↑	[95]
Cystain C	MCAO mice model	Activity of MMP-9↓	Expression↑	[96]
N-acetylcysteine	Diabetic MCAO mice model	Anti-oxidant and anti-inflammatory	Occludin glycation↑	[97]
1,25(OH)$_2$D$_3$	Hypoxia/reoxygenation mice model	ROS production and NF-κB activation	Expression↑	[99]
GC	MCAO mice model	Activation of the GR signaling pathway	Expression↑	[100]
Immunoglobulin	OGD mice model	Reducing leukocyte filtration	Expression↑	[101]
MiR-210-LNA	Hypoxic-Ischemic rats model	Unknown	Expression↑	[102]
LOC102640519	MCAO/R-injured mice model	VEGF-induced HOXC13 activity increased	Expression↓	[103]
Ischemic preconditioning	MCAO rats model	Activation of ERK1/2	Expression↑	[104]
Sphingosine kinase-2	HPC model	Generation of S1P	Expression↑	[105]

NBO: Normobaric hyperoxia, MMP: Matrix metalloproteinase, tMCAO: Transient middle cerebral artery occlusion, OGD/R: Oxygen-glucose deprivation/recovery, NVU: Neurovascular unit, NF-κB: Nuclear factor κB, PKC: Protein kinase C, PI3K: Phosphatidylinositol 3-kinase, GC: Glucocorticoid, GR: Glucocorticoid receptor, ROS: Reactive oxygen species, HPC: Hypoxic preconditioning-induced cerebral, SP1: Sphingosine-1-phosphate, GSK3: Glycogen synthase kinase 3, MiR-210-LNA: Microrna-210 complementary locked nucleic acid oligonucleotides, ERK: Extracellular signal-regulated kinase, VEGF: Vasclualr endothelial cell growth factor, TNF-α: Tumor necrosis factor-α, IL-1β: Interleukin-1β. Nox2: Nicoamide adenmine dinucleotide phosphooxidase

the expression level of occludin. Occludin degradation has been considered as a subsequent event driver of stroke, including brain edema and hemorrhagic transformation. Therefore, occludin might be a potential biomarker for early hemorrhagic transformation in ischemic stroke. There are also numerous ischemic stroke treatments targeting BBB via occludin, which are summarized in Table 2. Moreover, clinical evidence suggests that NBO is a promising approach to expand the time window of reperfusion therapies in ischemic stroke. Although occludin has been shown to play a critical role in regulating BBB integrity, more preclinical studies are required to elucidate the roles of occludin before it can be considered a viable therapeutic target for ischemic stroke.

Financial support and sponsorship

Conflicts of interest

There are no conflicts of interest.

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