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Nitric Oxide Interacts with Caveolin-1 to Facilitate Autophagy-Lysosome-Mediated Claudin-5 Degradation in Oxygen-Glucose Deprivation-Treated Endothelial Cells

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Abstract

Using in vitro oxygen-glucose deprivation (OGD) model, we have previously demonstrated that 2-h OGD induces rapid, caveolin-1-mediated dissociation of claudin-5 from the cellular cytoskeletal framework and quick endothelial barrier disruption. In this study, we further investigated the fate of translocated claudin-5 and the mechanisms by which OGD promotes caveolin-1 translocation. Exposure of bEND3 cells to 4-h OGD, but not 2-h OGD plus 2-h reoxygenation, resulted in claudin-5 degradation. Inhibition of autophagy or the fusion of autophagosome with lysosome, but not proteasome, blocked OGD-induced claudin-5 degradation. Moreover, knockdown of caveolin-1 with siRNA blocked OGD-induced claudin-5 degradation. Western blot analysis showed a transient colocalization of caveolin-1, claudin-5, and LC3B in autolysosome or lipid raft fractions at 2-h OGD. Of note, inhibiting autophagosome and lysosome fusion sustained the colocalization of caveolin-1, claudin-5, and LC3B throughout the 4-h OGD exposure. EPR spin trapping showed increased nitric oxide (NO) generation in 2-h OGD-treated cells, and inhibiting NO with its scavenger C-PTIO or inducible nitric oxide synthase (iNOS) inhibitor 1400W prevented OGD-induced caveolin-1 translocation and claudin-5 degradation. Taken together, our data provide a novel mechanism underlying endothelial barrier disruption under prolonged ischemic conditions, in which NO promotes caveolin-1-mediated delivery of claudin-5 to the autophagosome for autophagy-lysosome-dependent degradation.

Keywords

Oxygen-glucose deprivation; Claudin-5; Caveolin-1; Autophagy; Lysosome

Introduction

Degradation of tight junction (TJ) proteins is a decisive step in hypoxic blood-brain barrier (BBB) breakdown in stroke [1]. Claudins are transmembrane proteins that are the main constituents of the TJs [2]. Although the claudin superfamily consists of 24 membranes, claudin-1 and claudin-5 express primarily in the endothelial cells of mammalian capillaries, especially in the endothelium of the brain capillaries [3]. At the BBB, claudin-5 is a key molecule forming TJ strands to seal the intercellular space and maintain the function of the paracellular barrier [4–6]. While a growing body of data demonstrates that claudin-5 degradation occurs at the compromised BBB following cerebral ischemia and reperfusion [7–9], the underlying mechanisms remain to be understood.

Matrix metalloproteinases (MMPs), particularly MMP-2/9, have been shown to contribute to claudin-5 degradation under ischemic conditions [8–10]. However, whether claudin-5 is a direct substrate of MMP-2/9 remains debatable because incubation of endothelial extracts with purified MMP-2 or MMP-9 fails to induce claudin-5 degradation [11, 12] and MMP-independent claudin-5 degradation is reported in hypoxic conditions [8, 13]. More recently, claudin-5 has been shown to be polyubiquitinated on lysine 199, which triggers proteasome-dependent degradation [14]. In addition, the autophagy-lysosome pathway has been suggested to be involved in claudin-5 degradation [14, 15]. These studies suggest that claudin-5 degradation is a multifactorial process and may be stimulus dependent.

Using an in vitro model of ischemia, we recently showed that exposure of the endothelial monolayer to 2-h oxygen and glucose deprivation (OGD) led to rapid accumulation of claudin-5 in the cytosol, but no claudin-5 degradation was observed [16]. Moreover, in this process, caveolin-1 (Cav-1) trafficking was found to play an important role [16]. Our study has raised two important questions: (1) what is the intracellular fate of translocated claudin-5 if OGD continues, and 2) what triggers rapid translocation of Cav-1 under hypoxic-ischemia conditions?

The present study was aimed to answer these questions by examining the changes of claudin-5 and Cav-1 in mouse brain microvascular endothelial cells (bEND3 cells) after short (2 h) or prolonged (4 h) exposure to OGD or OGD followed by reoxygenation. Our data showed that claudin-5 was degraded via the autophagy-lysosome pathway after prolonged exposure to OGD, and inducible nitric oxide (NO) synthase (iNOS)-derived NO was the trigger for rapid Cav-1 translocation under ischemic conditions.

Results

OGD Induces Biphasic Changes in Claudin-5 in bEND3 Cells

We recently showed that exposure of bEND3 cells to OGD for 2 h led to cytosolic translocation of claudin-5, but its degradation did not occur at this early ischemic stage [16]. In view of the biphasic pattern of BBB injury under experimental stroke [17, 18] and the fundamental role of claudin-5 in maintaining BBB integrity, we speculated that claudin-5 may also respond to ischemia in a biphasic pattern, and if ischemia continues, claudin-5

might undergo degradation at a certain time point. To test this possibility, we extended OGD duration from 2 to 4 h. Right after OGD exposure, claudin-5 contents in the total cellular extracts and subcellular fractions were analyzed by Western blot. Consistent with our previous report [16], 2-h OGD did not affect total claudin-5 protein levels, but promoted its translocation from the actin cytoskeleton fraction (ACF) to the cytosolic fraction (CF) and the membranous fraction (MF) (Fig. 1a, b). When OGD was prolonged to 4 h, claudin-5 degradation occurred, as reflected by the reductions in total claudin-5 protein levels (Fig. 1a) and in the CF and MF, compared to their levels measured at 2-h OGD (Fig. 1b). To exclude the possibility that 2-h OGD may have already initiated the degrading process and this process will continue even if OGD discontinues, we exposed the cells to 2-h OGD plus 2-h reoxygenation. Indeed, claudin-5 degradation did not occur under this experimental condition (Fig. 1a), and no further change was observed for its subcellular distribution (Fig. 1b). These results indicate that prolonged ischemia is required for claudin-5 degradation.

Thus, ischemia, depending on its duration, could induce claudin-5 redistribution or degradation. We next tested if these changes could recover during prolonged reoxygenation. As shown in Fig. 1c, d, 2-h OGD-induced alternations in claudin-5 distribution were completely recovered at 24 h following reoxygenation, while reduced protein levels of claudin-5 were clearly seen in total cellular extracts and the ACF of 4-h OGD-treated cells (Fig. 1c, d). Taken together, these data indicate that prolonged ischemia may induce a biphasic change in claudin-5 protein, involving early subcellular redistribution and subsequent degradation, and claudin-5 degradation is more difficult to recover than that of redistribution.

Autophagy-Lysosome Pathway Is Involved in OGD-Induced Claudin-5 Degradation

We next investigated the mechanisms underlying OGD-induced claudin-5 degradation in bEND3 cells. The ubiquitin-proteasome and autophagy-lysosome pathways are the two main routes of intracellular protein clearance in eukaryotic cells [19]. To determine their involvement in OGD-induced claudin-5 degradation, we applied pharmacological approaches to inhibit proteasomes and lysosomes at 1 h before and during OGD treatment. Western blot showed that the lysosome inhibitor chloroquine (100 μ M), but not the proteasome inhibitor MG132 (20 μ M), blocked claudin-5 degradation in bEND3 cells exposed to 4-h OGD (Fig. 2a, b). To further verify the effect of chloroquine, we conducted immunostaining and examined claudin-5 protein change under a confocal microscope. Consistent with our previous report [16], control bEND3 cells exhibited a circumcellular immunostaining for claudin-5 (Fig. 2c). Exposure of bEND3 cells to 4-h OGD significantly reduced claudin-5 staining, and this reduction was completely inhibited by chloroquine (Fig. 2c). Of note, chloroquine itself did not affect claudin-5 distribution in control bEND3 cells, but its presence increased claudin-5 staining in the cytosol of OGD-treated cells (Fig. 2c), a distributing pattern comparable to what we observed for 2-h OGD-treated cells in a previous study [16], indicating that lysosome inhibition might not affect OGD-induced early claudin-5 redistribution. To test this possibility, we assessed claudin-5 levels in different subcellular fractions collected from 2 to 4-h OGD-treated cells with the presence of chloroquine. Indeed, we found that chloroquine did not affect the translocation of claudin-5 from ACF (reduction) to CF and MF (increase) at 2-h OGD, but it inhibited 4-h OGD-

induced claudin-5 reduction in the CF and MF (Fig. 2d). These results indicate that a lysosome-dependent pathway is involved in prolonged ischemia-induced claudin-5 degradation in endothelial cells.

Autophagy is an evolutionarily conserved process which delivers cytoplasmic constituents to the lysosome for degradation or catabolism [20], and is activated by various types of stress including ischemia [21]. To demonstrate a role of autophagy in mediating OGD-induced claudin-5 degradation, we first assessed the conversion of microtubule-associated protein light chain 3B-I (LC3B-I) to LC3B-II, a well-established indicator of autophagy activation [22]. As shown in Fig. 3a, increased conversion of LC3B-I to LC3B-II was seen in bEND3 cells at 2-h OGD, and when OGD was prolonged to 4 h, the conversion was significantly greater. Moreover, along with the conversion of LC3B-I to LC3B-II, total LC3B contents were also significantly increased in OGD-treated cells (Fig. 3a). To further establish a causal link between autophagy and claudin-5 degradation, we applied autophagy inhibitors bafilomycin A1 (BFA1, 100 nM) and 3-methyladenine (3-MA, 5 mM) that were added to the cells 1 h before and during 4-h OGD exposure. BFA1 has been widely used to inhibit autophagosome and lysosome fusion [23], while 3-MA acts as an autophagy inhibitor due to its inhibition on class III PI3K activity [24]. As shown in Fig. 3b, c, both agents completely blocked claudin-5 degradation induced by 4-h OGD. These data suggest that the autophagy-lysosome-dependent pathway mediates OGD-induced claudin-5 degradation.

An important step in autophagy-lysosome degradation pathway is the formation of autophagosome, a double-membrane-bounded structure that encloses cytoplasmic components and delivers them to the lysosome for degradation [25]. At 2-h OGD, we previously detected Cav-1 (probably caveolae)-mediated translocation of claudin-5 to the cytosol [16], where they might be engulfed as a caveolae/claudin-5 complex by autophagosome and then be delivered to the lysosome for degradation. If this is the case, there should be a transient localization of caveolae/claudin-5 in the autophagosome. To test this possibility, we isolated autophagosome from cells exposed to 2- or 4-h OGD using OptiPrep gradients [26] and examined the colocalization of LC3B, Cav-1, and claudin-5 in each gradient fraction using Western blot. For control cells, low basal level of LC3B was detected in fraction 2, and no Cav-1 and claudin-5 were detected in any of the gradient fractions (Fig. 4a, upper panel). After 2 h of OGD treatment, LC3B levels were significantly increased in fractions 2, 3, and 4, and interestingly, increased Cav-1 and claudin-5 levels were also detected in these fractions (Fig. 4a, middle panel). When OGD was prolonged to 4 h, the levels of LC3B remained high, but Cav-1 and claudin-5 completely disappeared from these fractions (Fig. 4a, bottom panel). These results indicate that there is a transient colocalization of Cav-1, claudin-5, and LC3B in 2-h OGD-treated bEND3 cells.

To provide further evidence supporting this transient colocalization, we isolated caveolin-enriched fractions using sucrose gradient (5–40 %) and detected Cav-1, claudin-5, and LC3B in collected gradient fractions using Western blot. Consistent with a previous study [27, 28], Cav-1 was found to be enriched in fractions 3, 4 and 5, where claudin-5 and LC3B were barely detectable under control condition (Fig. 4b). Following 2-h OGD treatment, increased localization of claudin-5 and LC3B was detected in caveolin-enriched fractions, particularly in fractions 4 and 5 (Fig. 4b). When OGD was prolonged to 4 h, claudin-5 and

LC3B vanished from these fractions (Fig. 4b). Of note, if autophagosome-lysosome fusion was inhibited by BFA1, claudin-5 and LC3B were consistently detectable in caveolin-enriched fractions throughout 4 h of OGD treatment (Fig. 4b). Taken together, these data indicate that the autophagy-lysosome pathway mediates claudin-5 degradation induced by 4-h OGD.

Cav-1-Mediated Claudin-5 Redistribution Is Required for Subsequent Degradation in bEND3 Cells Exposed to Prolonged OGD

In response to OGD, claudin-5 underwent early (2-h OGD) redistribution and late (4-h OGD) degradation. However, it was not clear whether these two changes are causally connected. Using the small interfering RNA (siRNA) approach to knock down Cav-1, we previously showed that OGD-induced early redistribution of claudin-5 was mediated by Cav-1 [16]. Therefore, we applied the same siRNA approach to test whether Cav-1-mediated early claudin-5 redistribution was required for its subsequent degradation at 4-h OGD. Consistent with our previous study [16], knockdown of Cav-1 with siRNA blocked 2-h OGD-induced translocation of claudin-5 from the ACF to the MF and CF (Fig. 5a, b). Importantly, when Cav-1 was knocked down, loss of claudin-5 in the CF (Fig. 5b) and total cellular extracts (Fig. 5c) were completely prevented in bEND3 cells exposed to 4-h OGD. These results suggest that Cav-1-mediated cytosolic translocation may be the first step for claudin-5 degradation under prolonged ischemic conditions.

Nitric Oxide Mediates OGD-Induced Cav-1 Translocation and Claudin-5 Degradation

Our data have shown that Cav-1 trafficking plays a critical role in OGD-induced claudin-5 redistribution [16] and degradation (Fig. 5). Then, what triggers this trafficking in OGD-treated bEND3 cells? S-nitrosylation of Cav-1 by NOS-derived NO has been shown to regulate Cav-1 trafficking [29]. Therefore, we speculated that NO might be an important trigger for OGD-induced Cav-1 redistribution. To test this possibility, we first assessed NO production and its enzymatic source. Using the well-established electron paramagnetic resonance (EPR) spin trapping technique [30], we showed that the EPR spectrum typical of NO was consistently detectable in the supernatant from the cells exposed to 2-h OGD, but not in control cells, indicating increased NO production in OGD-treated cells (Fig. 6a). There are two enzymatic sources of NO in endothelial cells, endothelial nitric oxide synthase (eNOS), and iNOS. Following 2 h of exposure to OGD, we found that iNOS messenger RNA (mRNA) and protein expression were significantly increased in bEND3 cells (Fig. 6b, c). Consistent with its nature of being a constitutively expressed enzyme [31], eNOS did not show any detectable changes in its expression following OGD treatment (data not shown). Therefore, iNOS appeared to be the enzymatic source for the increased NO production during OGD. This was further supported by the finding that the selective iNOS inhibitor 1400W (1 μ M) completely abolished the EPR signal of NO in OGD-treated cells (Fig. 6a).

Next, to establish a causal role of NO in Cav-1 translocation, we applied pharmacologic approaches to inhibit NO production or eliminate NO using its scavenger. As shown in Fig. 7a, b, inhibition of iNOS with 1400W (1 μ M) or scavenging NO with carboxy-PTIO (C-PTIO, 10 μ M) completely blocked OGD-induced Cav-1 translocation. To further support

this finding, we performed immunocytochemical analysis and the confocal micrographs showed that C-PTIO completely blocked Cav-1 accumulation in the cytosol observed for 2-h OGD (Fig. 7c), supporting that iNOS-derived NO was the important trigger for Cav-1 translocation under OGD conditions.

Lastly, we tested whether NO inhibition could prevent OGD-induced claudin-5 degradation. As shown in Fig. 8a, b, pretreatment of bEND3 cells with the iNOS inhibitor 1400W or the NO scavenger C-PTIO completely inhibited the reduction in total protein level of claudin-5 in 4-h OGD-treated cells. Taken together, these data indicate that iNOS-derived NO serves as the important trigger for OGD-induced Cav-1 trafficking and critically contributes to OGD-induced claudin-5 degradation.

Discussion

Degradation of the tight junction proteins is a decisive step in BBB breakdown during ischemic stroke [1]. In a previous study, we showed that exposure of endothelial cells to OGD induced rapid cytosolic translocation of caveolin-1, which mediated the dissociation of claudin-5 from the cytoskeleton to the cytosol [16]. In this study, we further investigated the fate of translocated claudin-5 under prolonged OGD conditions and the mechanisms underlying OGD-induced caveolin-1 redistribution. The major findings are as follows: (1) claudin-5 is degraded following 4-h OGD treatment, which is mediated by the autophagy-lysosome pathway; (2) caveolin-1/caveolae appears to serve as transcytotic vesicles to deliver claudin-5 to autophagosome for degradation by lysosome under prolonged OGD condition; and (3) iNOS-derived NO is the important trigger for promoting the rapid translocation of caveolin-1 to the cytosol in response to OGD.

Claudins and occludin are two key transmembrane proteins that form the seal of the tight junctions at the BBB [4–6]. Unlike occludin, claudin-5 is unlikely a direct substrate of MMPs [8, 13], and its changes appeared to be more complicated (redistribution and degradation) than occludin (only degradation) in the ischemic brain [9]. We have recently shown that 2-h OGD induces rapid translocation of claudin-5, but not degradation, in brain microvascular endothelial cells [16]. The data here show that claudin-5 was degraded when OGD was prolonged to 4 h, while no degradation was seen for 2-h OGD that was followed by 2-h reoxygenation, indicating that prolonged ischemia is required for claudin-5 degradation. Of note, once degradation occurs, ischemia-induced claudin-5 alternation appears to be difficult to recover, as after 24 h of reoxygenation, there is still a significant reduction of claudin-5 protein in the cytoskeletal fraction of 4-h OGD-treated cells, while claudin-5 redistribution induced by 2-h OGD is completely recovered. Here, we did not further investigate how long it may need for claudin-5 degradation to be covered and how this recovery correlates to the function of the endothelial barrier.

The ubiquitin-proteasome system and the autophagy-lysosome pathway are the two most important mechanisms that normally repair or remove abnormal proteins to maintain intracellular homeostasis [32]. Both pathways have been reported to mediate claudin-5 degradation in previous studies. For example, inhibiting autophagy with wortmannin attenuates nanoalumina-induced claudin-5 reduction, but not occludin disruption, in cultured

brain endothelial cells [33]. In another study, the ubiquitin-proteasome system is reported to be the major machinery responsible for claudin-5 degradation [14]. Our data here support an autophagy-lysosome-dependent degradation of claudin-5 in response to prolonged OGD because the inhibitors of autophagy or lysosome, but not of proteasome, blocked OGD-induced claudin-5 degradation.

Caveolin-1 is an essential structural constituent of caveolae [34], which buds into the cell with 50–100 nm plasma membrane invaginations observed in endothelial and epithelial cells [35]. Although caveolae was initially discovered to be associated with endocytosis [36, 37], accumulating evidence has implicated an important role of caveolae in protein trafficking [28, 33]. We have previously shown that 2-h OGD induces claudin-5 redistribution, which is mediated by caveolin-1. Our data here further demonstrate that knockdown of caveolin-1 with siRNA can also prevent the degradation of claudin-5 under prolonged ischemic conditions. Moreover, we observed a transient colocalization of caveolin-1, claudin-5, and LC3B in autophagosome-enriched fractions and the lipid raft fractions at 2-h OGD. When OGD is prolonged to 4 h, claudin-5 protein disappears from these fractions. Of note, inhibiting the fusion of lysosome and autophagosome with BFA1 sustains the colocalization of caveolin-1, LC3B, and claudin-5 throughout the 4-h OGD treatment. These data support that caveolin-1/caveolae may serve as transcytotic vesicles to deliver claudin-5 to the autophagosome for subsequent degradation by lysosome. It is worth pointing out that caveolin-1 protein also disappeared in autophagosome-enriched fractions at 4 h of OGD; however, we did not observe a reduction of total caveolin-1 protein levels in OGD-treated endothelial cells (data not shown). Caveolin-1 has been suggested to normally traffic to and from the cytoplasmic surface of lysosomes, while its degradation rate does not alter under various stress conditions [38]. Therefore, under OGD conditions, caveolin-1-mediated delivery of claudin-5 to autophagosome may be accelerated, while its degradation at the lysosome remains unchanged. Future studies are warranted to test this possibility.

Lastly, we investigated the molecules triggering caveolin-1 redistribution under OGD conditions. NO has garnered our attention because it has been reported to regulate caveolin-1 through direct nitration [39], S-nitrosylation [29], or indirectly interfering with its ubiquitination [40]. Anoxia/reoxygenation increases iNOS-derived NO generation in hippocampal slices [41]. Similarly, here, our data show that OGD increases NO generation in endothelial cells via upregulating iNOS expression. More importantly, we found that the iNOS inhibitor or NO scavenger completely blocks OGD-induced caveolin-1 translocation as well as the degradation of claudin-5, revealing a novel mechanism of NO in contributing to ischemic endothelial barrier injury. S-nitrosylation of caveolin-1 has been shown to regulate its trafficking [29]. However, future studies are needed to determine whether this mechanism is responsible for the increased cytosolic translocation of caveolin-1 under ischemic conditions.

Although our data clearly demonstrate that OGD induces a biphasic change of claudin-5 and the underlying mechanisms in vitro, the use of the transformed cell line bEND3 could be a caveat because using in vitro endothelial cultures may not assure similar changes occurring in in vivo stroke models and it may also be different from primary cultures. In the literature, several studies have shown a biphasic pattern of BBB damage in ischemic stroke [17, 42,

43]; however, the mechanism underlying the phenomenon remains unclear. Our data here may raise an important mechanistic possibility that redistribution of claudin-5 may contribute to the first phase of BBB damage (reversible), while degradation of claudin-5 may be the mechanistic basis and the hallmark of the second phase of BBB damage (irreversible). This hypothesis is currently under testing in our lab.

Taken together, our present study has shown for the first time that NO, caveolin-1, and autophagosome interact with each other to induce claudin-5 degradation under prolonged ischemic conditions, in which NO acts as a trigger to promote the delivery of claudin-5 by caveolae/ caveolin-1 to autophagosome for subsequent degradation by lysosome.

Materials and Methods

Cell Cultures

Mouse brain microvascular endothelial cells bEND3 (American Type Culture Collection) were grown as a monolayer in Dulbecco's modified Eagle's medium (DMEM) with 15 % fetal bovine serum (FBS), 100 U/ml penicillin, and 100 µg/ml streptomycin at 37 °C in a humidified incubator with 5 % CO₂ and 95 % room air. The cells were grown to confluence on type I collagen-coated 60-mm dishes before exposure to OGD. To verify the role of lysosome and autophagy in claudin-5 degradation, two lysosome inhibitors BFA1 (100 nM, Sigma) and chloroquine (100 µM, Sigma), and the autophagy inhibitor 3-MA (5 mM, EMD) were added to cells 1 h before and during OGD treatment. To demonstrate the role of iNOS-derived NO in OGD-induced Cav-1 redistribution, the selective iNOS inhibitor 1400W (1 µM, Cayman) and the NO scavenger C-PTIO (10 µM, Cayman) were applied.

OGD Treatment

bEND3 cells were exposed to OGD as we described previously [16]. In brief, confluent bEND3 cells were subjected to OGD by replacing the normal growth medium with glucose-free medium (DMEM without glucose) pre-equilibrated with 95 % N₂ and 5 % CO₂. Cells were then incubated in a humidified airtight chamber (Billups-Rothberg Inc.) for 2 or 4 h. Control cultures were incubated with normal DMEM medium without FBS at 37 °C in 5 % CO₂/95 % air. Immediately after OGD treatment, the conditioned media (CM) and cells were collected separately for further analyses. Under these experimental conditions, OGD did not induce noticeable cytotoxicity assessed by measuring lactate dehydrogenase (LDH) release using a CytoTox 96® Non-Radioactive Cytotoxicity Assay Kit (Promega) (data not shown).

Measurement of NO with EPR Spectroscopy

At the end of each indicated treatment, 0.5 ml CM was collected for NO detection with EPR as described previously [44, 45]. In brief, the NO spin trap Fe(MGD)₂ was freshly prepared by mixing a stock solution of ferrous sulfate and NaMGD (Enzo) in a molar ratio of 1:5 in distilled water, anaerobically, prior to each experiment. Right after the addition of 1 mM Fe(MGD)₂, bEND3 cells (2×10⁶) were exposed to OGD or normoxia for 2 h at 37 °C with or without the presence of the iNOS specific inhibitor 1400W (1 µM, Cayman). At the end of exposure, 0.5 ml CM were immediately transferred into custom-made gas permeable

Teflon tubing (Zeus Industries), folded four times, and inserted into a quartz EPR tube for measuring NO spectrum using Bruker EleXsys 540 X-band EPR spectrometer (Billerica, MA) operating at 9.03 GHz and 100 kHz field modulation and spectra. Instrument settings were as follows: magnetic field, 3443 G; scan range, 100 G; microwave power, 21 mW; modulation frequency, 100 kHz; modulation amplitude, 1.0 G; and time constant, 20 ms. The EPR spectra were collected, stored, and manipulated using the Bruker Software Xepr (Billerica, MA).

Preparation of Total Cell Lysate and Subcellular Fractions

After OGD treatment, total cellular lysates and three subcellular fractions including MF, CF, and actin ACF were extracted using RIPA buffer (Santa Cruz Biotech) or ProteoExtract Subcellular Proteome Extraction Kit (Calbiochem), respectively, as we described previously [16]. The extraction of sub-cellular fractions was briefly described as follows: after OGD treatment, the cell culture medium was carefully removed without disturbing the cell monolayer, and the cells were washed twice with 2 ml ice-cold wash buffer. Then, the cells were incubated with 1 ml ice-cold extraction buffer I containing 1× protease inhibitor cocktail for 10 min at 4 °C under gentle agitation. The supernatant (CF) was transferred to a clean tube and stored on ice. The cells without CF were incubated with 1 ml ice-cold extraction buffer II containing 1× protease inhibitor cocktail for 30 min at 4 °C under gentle agitation. The supernatant (MF) was transferred to a clean tube and stored on ice. The cells without CF and MF were incubated with 500 µl ice-cold extraction buffer III containing 1× protease inhibitor cocktail and 1.5 µl (375 U) Benzonase® nuclease for 10 min at 4 °C under gentle agitation. The supernatant (nuclear fraction) was transferred to a clean tube and stored on ice. The cells without CF, MF, and nuclear fraction were solubilized in 500 µl extraction buffer IV containing 1× protease inhibitor cocktail, and the extracts (ACF) were transferred to a clean tube and stored on ice. Each fraction was verified by detecting their specific marker using Western blot, cytochrome CYPOR for MF, calpain for CF, histone 1 for nuclear fraction, and vimentin for ACF in our previous study [16], and we did not repeat this verification experiment here. Because OGD treatment did not change the levels of Cav-1 and claudin-5 in the nuclear fraction of bEND3 cells, this fraction was not used for further analysis.

siRNA Transfection

bEND3 cells at 60–70 % confluence were transfected with 80 pmol of Cav-1 siRNA (Santa Cruz, sc-29520) or scrambled control siRNA (Santa Cruz Biotech, sc-37007) using siRNA Transfection Reagent (Santa Cruz Biotech) according to the manufacturer's instruction. Forty-eight hours after transfection, cells were subjected to OGD treatment. Specific silencing was confirmed by Western blot.

Isolation of Lysosome

The lysosome and autolysosome were isolated from bEND3 cells using a lysosome isolation kit (Sigma-Aldrich) according to manufacturer's protocol. In brief, after OGD treatment, the cells grown on 150 mm dishes were collected and homogenized in 2.7 ml extraction buffer containing protease inhibitor cocktail (Sigma). After an initial centrifugation at 1000g for 10 min, the supernatants were further centrifuged at 20,000g for 20 min to collect the crude

lysosomal fraction (CLF) which contains mitochondria, lysosomes and autolysosome, peroxisomes, and endoplasmic reticulum. To further separate the lysosome from other organelles, the CLF was diluted to a solution containing 19 % OptiPrep density gradient medium, and then built up a step gradient with 27 % OptiPrep density gradient medium solution at the bottom and 8 % OptiPrep density gradient medium solution at the top. After centrifugation at 150,000g for 4 h, ten fractions of 1.0 ml were collected from the top to the bottom of the gradient and used for Western blot analysis.

Isolation of Caveolae-Enriched Fractions

To prepare the caveolae-enriched fractions, 1×10^8 bEND3 cells were lysed in 1 ml 1 % Triton X-100 in MNE buffer (25 mM MES, pH 6.5, 150 mM NaCl, 5 mM EDTA) in the presence of protease inhibitor cocktail [46]. The lysates were sonicated and centrifuged at 2000g for 10 min to remove nuclei. Clarified postnuclear supernatants were diluted 1:2 with 80 % (w/v) sucrose in MNE buffer, placed at the bottom of 12.5-ml ultracentrifuge tubes (Beckman), and overlaid gently with 6 ml of 35 % and 3 ml of 5 % sucrose. The resulting 5–40 % discontinuous sucrose gradient was centrifuged at 40,000 rpm for 20 h at 4 °C to separate the low-density caveolae. After centrifugation, 12 fractions (1.0 ml) were collected from the top to the bottom of the gradient and used for further analysis.

Western Blot

Cav-1, claudin-5, and iNOS in total cell lysates and subcellular fractions (CF, MF, and ACF) were analyzed by Western blot as we described previously [16]. In brief, protein samples were electrophoresed in SDS-PAGE acrylamide gels, transferred onto nitrocellulose membranes (Bio-Rad). After blocking with 5 % nonfat milk, membranes were incubated overnight at 4 °C with primary antibodies against Cav-1 (Santa Cruz Biotech, 1:500), claudin-5 (Invitrogen, 1:1000), or iNOS (Invitrogen, 1:500), followed by incubation with corresponding HRP-conjugated anti-rabbit or anti-mouse antibodies (Santa Cruz Biotech, 1:1000). The membranes were developed with the SuperSignal West Pico HRP substrate kit (Pierce) and photographed on a Kodak 4000 image station (Carestream Molecular Imaging). To control sample loading and protein transfer, the membranes were stripped and reprobed with β -actin antibody (Santa Cruz Biotech, 1:1000). For subcellular fraction samples, we noticed that the actin levels were comparable for each sample among different fractions, so we only used one normalizing loading control (CF actin) for all three subcellular fractions.

Immunocytochemistry of Claudin-5 and Cav-1

bEND3 cells grown on type I collagen-coated coverslips were exposed to OGD for 2 or 4 h before fixation with 4 % para-formaldehyde and permeabilization with 0.1 % Triton X-100. After blocking nonspecific binding with a blocking solution (3 % BSA, 0.1 % Tween 20, and 5 % goat serum in PBS), the cells were incubated overnight at 4 °C with anti-Cav-1 (Santa Cruz, 1:200) or anti-claudin-5 (Invitrogen, 1:100) primary antibodies, followed by 1-h incubation with FITC-conjugated anti-mouse (for claudin-5) or Cy3-conjugated anti-rabbit (for Cav-1) second antibodies (Invitrogen, 1:200) at room temperature. Coverslips were mounted on glass slides using anti-fade solution Vectashield (Vector Laboratories), and immunostaining was visualized under a LSM 510 confocal laser-scanning microscope (Zeiss).

Real-Time RT-PCR

Total cellular RNA was isolated using TRIzol reagents (Invitrogen). After reverse transcription using TaqMan[®] Reverse Transcription Kits (Applied Biosystems), 0.5 µl reverse-transcribed products were amplified with the 7900HT real-time PCR System (Applied Biosystems) in a 10-µl final reaction volume using SYBR[®] Green PCR Master Mix (Applied Biosystems) under the following conditions: 2 min at 50 °C and 10 min at 95 °C, followed by a total of 40 cycles of two temperature cycles (15 s at 95 °C and 1 min at 60 °C). Primers (purchased from IDT) for iNOS (NM_010927) were forward: 5'-GGGCTGTACGGAGATCA-3' and reverse: 5'-CCATGATGGTCACATTCTGC-3'. GAPDH (NM_008084) served as the endogenous control, and the primers were forward: 5'-CAATGTGTCCGTCGTGGATCT-3' and reverse: 5'-GTCCTCAGTGTAGCCCAAGATG-3'. The fluorescence threshold value (C_t value) was calculated by the comparative C_t method using the SDS Enterprise Database software (Applied Biosystems).

Statistical Analysis

All data were expressed as means ± SEM. Differences between groups were evaluated by either an unpaired Student's *t* test or one-way ANOVA followed by Tukey's post hoc test as indicated in the figure legends. A value of *P* 0.05 was considered statistically significant.

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Compliance with Ethical Standards

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Fig. 1.

Exposure of bEND3 cells to 4-h OGD induces and causes the degradation of claudin-5. The bEND3 cells were subjected to the following treatments: 2-h OGD (*O2h*), 2-h OGD plus 2-h reoxygenation (*O2R2*), 4-h OGD (*O4h*), 2-h OGD plus 24-h reoxygenation, and 4-h OGD plus 24-h reoxygenation before collecting total cell lysates or sub-cellular fractions for Western blot analysis. **a** A significant reduction in claudin-5 protein levels was detected in bEND3 cells exposed to *O4h*, but not *O2h* or *O2R2*. *Upper panel*: representative immunoblots for claudin-5 and β -actin; *bottom panel*: quantitative data of claudin-5 protein band intensity after normalization to β -actin. Data were expressed as mean $\pm$ SEM, $n=4$, * P < 0.05 versus control (*Ctrl*), ANOVA. **b** Representative immunoblots showed that the claudin-5 contents in the cytosolic fraction (*CF*), membranous fraction (*MF*), and actin cytoskeleton fraction (*ACF*) were significantly reduced in *O4h*-treated cells, and there was a redistribution of claudin-5 in *O2h*- or *O2R2*-treated cells, with reduced protein levels in the *ACF* and increased protein levels in the *CF* and *MF*. Experiments were repeated 3 times with similar results. **c** After 24 h of reoxygenation, the total protein level of claudin-5 remained

significantly reduced in O4h-treated bEND3 cells when compared with control cells or O2h-treated cells. * $P < 0.05$ versus Ctrl, ANOVA; $n = 4$. **d** Representative immunoblots showed that after 24-h reoxygenation, the reduction of claudin-5 was recovered in CF and MF, but not in ACF of O4h-treated cells; however, O2h-induced redistribution of claudin-5 was completely recovered. Experiments were repeated 3 times with similar results

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Fig. 2.

Lysosome pathway is responsible for claudin-5 degradation in 4-h OGD-treated endothelial cells. bEND3 cells were treated with lysosome inhibitor chloroquine (100 μ M) or proteasome inhibitor MG132 (20 μ M) 1 h before and during 4-h OGD treatment (O4h) before collecting indicated cellular extracts for claudin-5 detection by Western blot. The degradation of claudin-5 was blocked by chloroquine (a), but not MG132 (b), in O4h-treated cells. *Upper panels*: representative immunoblots for claudin-5 and β -actin; *bottom panels*: quantitative data of claudin-5 protein band intensity after normalization to β -actin. Data were expressed as mean $\pm$ SEM, $n=5$ for chloroquine and $n=4$ for MG132, $*P<0.05$ versus control (Ctrl)+vehicle (0.1 % DMSO), ANOVA. **c** Representative confocal microphotographs showed circumcellular staining of claudin-5 in control bEND3 cells

(*Ctrl*), which was significantly reduced after O4h treatment. The addition of chloroquine prevented claudin-5 reduction and resulted in its accumulation in the cytosol. Experiments were repeated 3 times with similar results. *Scale bar*, 40 μm . **d** Western blot analyses showed that 2-h OGD (*O2h*) induced the translocation of claudin-5 from the ACF to the CF and MF. When OGD was prolonged to 4 h (*O4h*), claudin-5 levels were decreased in all three subcellular fractions. The presence of chloroquine prevented claudin-5 degradation and rendered its redistribution comparable to that of O2h-treated cells. Experiments were repeated 3 times with similar results

Fig. 3.

Autophagy is involved in 4-h OGD-induced claudin-5 degradation in bEND3 cells. **a** A significant increase in the level of LC3B (both LC3B-I and LC3B-II), the marker of autophagosome, was detected in bEND3 cells exposed to 2-h OGD (O2h) or 4-h OGD (O4h), with a greater increase for O4h. Experiments were repeated 3 times with similar results. **b, c** bEND3 cells were treated with or without autophagosome-lysosome fusion inhibitor BFA1 (100 nM) and autophagy inhibitor 3-MA (5 mM) 1 h before and during O4h treatment before collecting total cellular extracts for claudin-5 detection by Western blot. Both inhibitors blocked O4h-induced claudin-5 degradation. β -Actin served as a loading control. Data were expressed as mean $\pm$ SEM, $n=4$, * $P<0.05$ versus control (Ctrl)+vehicle, ANOVA

Fig. 4.

There was a transient colocalization of LC3B, claudin-5, and Cav-1 in bEND3 cells exposed to 4-h OGD treatment. Following indicated treatments, OptiPrep (60 % w/v iodixanol in H₂O) gradients and 5–40 % discontinuous sucrose gradient were used to isolate autolysosome-enriched fractions or caveolins/caveolae-enriched fractions from bEND3 cells, respectively, and Cav-1, claudin-5, and LC3B in each fraction were analyzed by Western blot. **a** A transient colocalization of Cav-1, claudin-5, and LC3B was detected in autophagosome-enriched fractions (fractions 2, 3, and 4) after 2-h OGD treatment (*OGD2h*); when OGD was prolonged to 4 h (*OGD4h*), claudin-5 and Cav-1 disappeared from these fractions. **b** A similar colocalization of Cav-1, LC3B, and claudin-5 was transiently seen in caveolins/caveolae-enriched fractions (lipid raft, fractions 3, 4, 5) after 2-h OGD treatment. When OGD was prolonged to 4 h, the protein bands of claudin-5 and LC3B vanished from these fractions, while the presence of autophagosome-lysosome fusion inhibitor BFA1 (O4h +BFA1) sustained the colocalization of these three proteins. Both experiments were repeated for 3 times with similar results

Fig. 5.

Cav-1 is involved in OGD-induced claudin-5 degradation in bEND3 cells. bEND3 cells were incubated with control siRNA (Ctrl siRNA) or Cav-1 siRNA for 48 h before exposing to 2-h OGD (02h) or 4-h OGD (04h). **a** Western blot analysis showed Cav-1 siRNA effectively knocked down Cav-1 protein expression (~90 % reduction) in bEND3 cells compared to Ctrl siRNA. **b** Knockdown of Cav-1 with siRNA prevented O2h-induced claudin-5 redistribution (i.e., increased contents in the cytosolic and membranous fractions — CF and MF, and reduced contents in the actin cytoskeleton fraction — ACF) as well as O4h-induced claudin-5 reduction in all three subcellular fractions. Experiments were repeated 3 times with similar results. **c** Knockdown of Cav-1 with siRNA abolished O4h-induced claudin-5 degradation. *Upper panels*: representative immunoblots for claudin-5 and β -actin; *bottom panels*: quantitative data of claudin-5 protein band intensity after normalization to β -actin. Data were expressed as mean $\pm$ SEM, $n=5$, * $P<0.05$ versus Ctrl+Ctrl siRNA, ANOVA

Fig. 6.

Exposure to 2-h OGD increased iNOS-derived NO production in bEND3 cells. **a** bEND3 cells were incubated with spin trapping agent $\text{Fe}(\text{MGD})_2$ (1 mM) during 2-h OGD (*O2h*) treatment with or without the presence of iNOS specific inhibitor 1400W. At the end of exposure, NO in 0.5 ml media was immediately measured by EPR. A typical NO signal was detected in the media of O2h-treated cells, but not in normoxic cells, and the presence of iNOS inhibitor 1400W completely abolished NO's signal. Experiments were repeated for 3 times with the same results. **b, c** After treatment, total RNA and protein were extracted from bEND3 cells for real-time PCR and Western blot analysis, respectively. Comparing to

normoxia (control), OGD significantly increased iNOS mRNA (**b**) and protein expression (**c**). *Upper panels*: representative immunoblots for claudin-5 and β -actin; *bottom panels*: quantitative data of claudin-5 protein band intensity after normalization to β -actin. Data were expressed as mean $\pm$ SEM, $n=4$, * $P<0.05$ versus control, Student's t test

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Fig. 7. iNOS-derived NO mediated OGD-induced Cav-1 translocation in bEND3 cells. bEND3 cells were exposed for 2-h OGD (*O2h*) with or without the presence of NO scavenger C-PTIO and iNOS inhibitor 1400W before analyzing subcellular redistribution of Cav-1 by Western blot or immunostaining. Following OGD treatment, Cav-1 protein levels were significantly increased in the cytosolic fraction (*CF*), which was accompanied by a significant reduction in the actin cytoskeleton fraction (*ACF*) (**a**, **b**). Scavenging NO with C-PTIO (**a**) or inhibiting iNOS with 1400W (**b**) completely abolished these changes. *Upper*

panels: representative immunoblots of Cav-1 and β -actin; *bottom panel*: quantitative data of Cav-1 protein band intensity after normalization to β -actin. Data were expressed as mean $\pm$ SEM, $n=4$; * $P<0.05$ versus vehicle+control (*Ctrl*), ANOVA. **c** Confocal micrograph showed that 2-h OGD significantly increased Cav-1 immuno-staining in the cytosol, which was blocked by C-PTIO. Experiments were repeated 3 times with similar results. *Scale bar*, 40 μ m

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Fig. 8.

NO scavenger C-PTIO or iNOS inhibitor 1400W inhibited 4-h OGD-induced claudin-5 degradation in bEND3 cells. bEND3 cells were exposed to OGD treatment for 4 h (*O4h*) with or without the presence of C-PTIO or 1400W before collecting total cellular extracts for claudin-5 detection by Western blot. Both C-PTIO (**a**) and 1400W (**b**) inhibited claudin-5 degradation induced by 4-h OGD. *Upper panel*: representative immunoblots of claudin-5 and β-actin; *bottom panel*: quantitative data of claudin-5 protein band intensity after normalization to β-actin. Data were expressed as mean±SEM, $n=4$, * $P<0.05$ versus vehicle +control, ANOVA