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The effect of ASK1 on vascular permeability and edema formation in cerebral ischemia

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ABSTRACT

Apoptosis signal-regulating kinase-1 (ASK1) is the mitogen-activated protein kinase kinase kinase (MAPKKK) and participates in the various central nervous system (CNS) signaling pathways. In cerebral ischemia, vascular permeability in the brain is an important issue because regulation failure of it results in edema formation and blood–brain barrier (BBB) disruption. To determine the role of ASK1 on vascular permeability and edema formation following cerebral ischemia, we first investigated ASK1-related gene expression using microarray analyses of ischemic brain tissue. We then measured protein levels of ASK1 and vascular endothelial growth factor (VEGF) in brain endothelial cells after hypoxia injury. We also examined protein expression of ASK1 and VEGF, edema formation, and morphological alteration through cresyl violet staining in ischemic brain tissue using ASK1-small interference RNA (ASK1-siRNA). Finally, immunohistochemistry was performed to examine VEGF and aquaporin-1 (AQP-1) expression in ischemic brain injury. Based on our findings, we propose that ASK1 is a regulating factor of vascular permeability and edema formation in cerebral ischemia.

1. Introduction

Apoptosis signal-regulating kinase 1 (ASK1, also referred to as MAP3K5)(Ichijo et al., 1997) participates in many different stress responses, including apoptosis (Chang et al., 1998; Chen et al., 1999; Ichijo et al., 1997; Kanamoto et al., 2000; Noguchi et al., 2008; Saitoh et al., 1998; Tobiume et al., 2001; Wang et al., 1999; Wendt et al., 1994), cytokine secretion (Matsuzawa et al., 2005) and cell differentiation (Sayama et al., 2001; Takeda et al., 2000). ASK1 is activated in response to various stresses, including oxidative stress, endoplasmic reticulum (ER) stress (Hattori et al., 2009; Matsukawa et al., 2004; Takeda et al., 2003). Several studies have demonstrated that ASK1 overexpression induces apoptosis in various cell

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types (Chang et al., 1998; Saitoh et al., 1998). Ischemic stroke leads to disruption of the blood–brain barrier (BBB), which subsequently causes vasogenic edema (Unterberg et al., 2004) and cytotoxic edema (Loreto and Reggio, 2010; Nag et al., 2009; Simard et al., 2007), with the latter characterized as swelling of the astrocytes and neuronal dendrites (Risher et al., 2009). Cytotoxic edema occurs shortly after ischemic onset and is the results of translocation of interstitial water into the intracellular compartment (Betz et al., 1989; Young et al., 1987). Vasogenic edema disrupts cerebrovascular endothelial tight junctions, leading to increased permeability to albumin and other plasma proteins (Unterberg et al., 2004), and elevated intracranial pressure (Nag et al., 2009). Finally, vasogenic edema results in water accumulation in damaged brain areas (Nag et al., 2009; Yang and Rosenberg, 2011). Reperfusion after occlusion induces overpressure accompanied by shear stress (Hirt et al., 2009; Ribeiro et al., 2006) and leads to further entry of water through endothelial cells, resulting in brain swelling (Hirt et al., 2009; Ribeiro Mde et al., 2006) and further increases BBB permeability (Hirt et al., 2009; Strbian et al., 2008). According to previous studies, edema and cerebral infarction are especially exacerbated during ischemia/reperfusion (I/R) (Bleilevens et al., 2013). Hypoxic (low level of oxygen) and ischemic (low levels of oxygen and glucose) states caused by stroke also activate ASK1 (Bitto et al., 2010; Harding et al., 2010; Kwon et al., 2005). One study demonstrated that the increased ASK1 expression triggers apoptotic cell death after IR, whereas ASK1-small interference RNA (siRNA) attenuates ASK1 upregulation and reduces infarction in ischemic brain (Kim et al., 2011). Another study reported that anti-ASK1 short hairpin RNA (shRNA) suppresses ASK1 in the oxidative stress state induced by cerebral I/R (An et al., 2013). Several studies suggested that an ischemic state leads to dissociation of thioredoxin (Trx) from ASK1 by reactive oxygen species (ROS) generation and induces the activation of ASK1-mediated apoptosis pathways (e.g., the p38 pathway) (Ke and Costa, 2006). Activated p38 triggers the phosphorylation of hypoxia-inducible factor-1-a (HIF1-α), which modulates the expression of various target genes encoding proteins under hypoxic conditions (Ke and Costa, 2006; Kwon et al., 2005). Vascular endothelial growth factor (VEGF), an important HIF1-α target gene and vascular permeabilizing factor (Fischer et al., 1999; Minchenko et al., 1994) is induced by hypoxia and decreases the expression of BBB tight junction proteins (Keck et al., 1989), such as ZO-1 (Fischer et al., 2002; Yeh et al., 2007) and occludin(Fischer et al., 2002; Luissint et al., 2012). Furthermore, VEGF induces BBB disruption and vasogenic edema (Kimura et al., 2005; Roberts and Palade, 1995; Sood et al., 2008; van Bruggen et al., 1999; Wang and Tsirka, 2005) under ischemic stroke. Considering research into the role of ASK1 in ischemia-induced angiogenesis in vivo, ASK1 is involved in VEGF expression in ischemic tissue and promotes early angiogenesis by stimulating VEGF expression (Izumi et al., 2005). Aquaporin (AQP)-1, a family of water channels, is known as a water-selective transporting protein in cell membranes as CHIP28 (CHannel-like Integral membrane Protein of 28 kDa) (Agre et al., 1993; Smith and Agre, 1991). In hypoxic conditions, AQP-1 expression is upregulated in human endothelial cells (Kaneko et al., 2008). AQP-1

Gene Symbol	Gene Name	GeneBank Number	Fold Change (vs MCAO of si-con+MCAO)
Mmp3	matrix metallopeptidase 3	NM_010809	0.44
Aqp12	aquaporin 12	NM_177587	0.48
Vegfa	vascular endothelial growth factor A	NM_001025257	0.75
Itga8	integrin alpha 8	NM_001001309	0.36
Lamb3	laminin, beta 3	NM_008484	0.41
Gjb3	gap junction protein, beta 3	NM_001160012	0.38
Cdh1	cadherin 1	NM_009864	0.05
Lama4	laminin, alpha 4	NM_010681	0.30
Icam1	intercellular adhesion molecule 1	NM_010493	0.44
Nes	nestin	NM_016701	0.33
Gjb1	gap junction protein, beta 1	NM_008124	0.18
Aqp8	aquaporin 8	NM_007474	0.27
Vegfc	vascular endothelial growth factor C	NM_009506	0.80
Sele	selectin, endothelial cell	NM_011345	0.10
Itga2b	integrin alpha 2b	NM_010575	0.45

Fig. 1 – The association between ASK1 and vascular permeability-related genes. In microarray analysis of ischemic lesions at reperfusion 8 h after MCAO, vascular permeability-related genes were down-regulated in accordance with reduction of ASK1 by ASK1-siRNA. The genes, such as matrix metallopeptidase 3 (*MMP3*), integrin alpha 8 (*Itga8*), cadherin 1 (*Cdh1*), gap junction protein beta 1 (*Gjb3*), Selectin (*Sele*), intercellular adhesion molecule 1 (*Icam1*), aquaporin 8(*Aqp8*), aquaporin 12 (*Aqp12*) were decreased in the ASK1-siRNA treated MCAO group (< fold change 1.0), compared with MCAO group or si-control treated MCAO group. In selected genes, vascular endothelial growth factor A (*Vegfa*), and vascular endothelial growth factor C (*Vegfc*) were down-regulated less than 2 fold compared with MCAO group or si-control treated MCAO group. The fold changes were comparative values, which were the ratio of values in the ASK1-siRNA treatment MCAO group, relative to those in MCAO group.

activity is stimulated by hypertonicity and is regulated by ERK, p38, and JNK activation (Umenishi and Schrier, 2003) and is associated with stress-induced endothelial cell migration (Saadoun et al., 2005). In present study, we investigated whether ASK1 affects vascular permeability and edema formation after ischemic brain injury. We show that ASK1 inhibition is linked to the prevention of edema formation under hypoxic injury. Thus, our results suggest that ASK1 regulation might alleviate stroke-induced pathological alterations by protecting the disruption of BBB following cerebral ischemic injury.

2. Results

2.1. *ASK1-related gene screening using microarray analysis in MCAO injury brain*

To investigate whether ASK1 inhibition alters the expression of permeability-related genes, we performed microarray analyses (Fig. 1). We sorted genes that were increased over 2-fold in the MCAO group compared with normal group, then screened for genes that were down-regulated more than 2-fold in the si-ASK1 group compared with the MCAO group. Several genes were selected, including matrix metallopeptidase 3 (*MMP3*) (Ashina et al., 2010), integrin alpha 8 (*Itga8*) (Cucullo et al., 2011; Osada et al., 2011), cadherin 1 (*Cdh1*) (Zechariah et al., 2013), gap junction protein beta 1 (*Gjb3*) (Song et al., 2007), Selectin (*Sele*) (Jin et al., 2010), intercellular adhesion molecule 1 (*Icam1*) (An and Xue, 2009), aquaporin 8 (*Aqp8*) (Richard et al., 2003), aquaporin 12 (*Aqp12*) (Calvanese et al., 2013) related with vascular permeability. Also, vascular endothelial growth factor A (*Vegfa*) (Gong et al., 2014; Poittevin et al., 2014), and vascular endothelial growth factor C (*Vegfc*) (Foster et al., 2008; Xu et al., 2013) which are related with vascular permeability were down-regulated in the si-ASK1 group compared with the MCAO group slightly. Based on these results, we confirmed the vascular permeability genes related with ASK1 in ischemic injury brain.

2.2. *The alteration of VEGF and ASK1 protein levels after OGD/R*

We conducted western blot analysis to examine the protein level of ASK1 (Fig. 2A) and VEGF (Fig. 2B), which is known to play important roles in vascular permeability following OGD/R. This data shows the protein level in various reperfusion time points (reperfusion 0 min, 30 min, 1 h, and 3 h) after OGD (Fig. 2). VEGF protein expression was significantly increased at reperfusion 0 min after OGD. VEGF protein level was augmented from reperfusion 0 min to 30 min. However, they were gradually decreased from reperfusion 1–3 h after OGD (Fig. 2A). Western blotting was also performed to evaluate ASK1 expression in OGD/R injured bEND.3.cells (Fig. 2B). The protein level of ASK1 was highly augmented after hypoxia injury and especially peaked at reperfusion 30 min after OGD. ASK1 protein level was gradually decreased in bEND.3.cells from reperfusion 1–3 h after OGD. This result suggests that ASK1 may be associated with the expression of VEGF in brain endothelial cells after cerebral ischemia. Also,

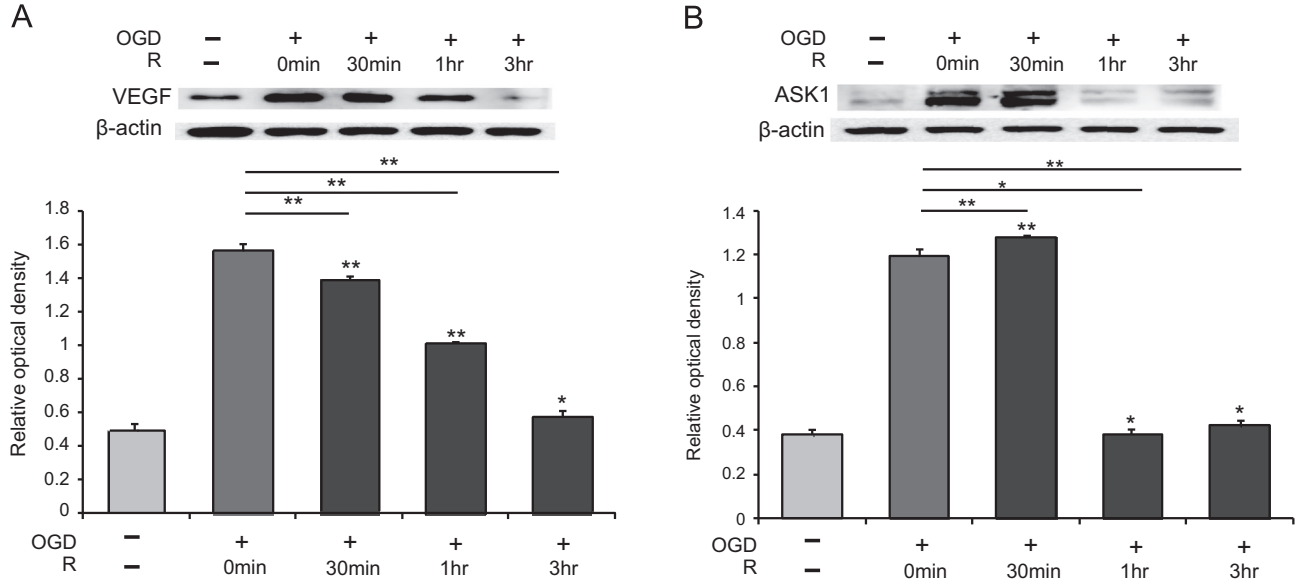

Fig. 2 – The change of VEGF and ASK1 protein level in bEnd.3.cells in different reperfusion time after OGD injury. (A) The protein level of VEGF was increased after OGD/R injury. After OGD/R, VEGF protein level was augmented compared with the normal control group. The protein level of VEGF was peaked at reperfusion 0 min after OGD injury. (B) The protein level of ASK1 was also increased after OGD/R injury. The protein level of ASK1 was peaked at reperfusion 30 min after OGD injury. The protein level of β-actin was used as an internal control. Data were expressed as mean ± S.E.M, and were analyzed statistically using one-way ANOVA, followed by Bonferroni's post hoc. Statistical significance with the OGD and reperfusion 0 min group was determined by t-test. Each experiment included at least three replicates per condition. Differences were considered significant at $^{*}p<0.05$, $^{**}p<0.01$.
OGD: **OGD 4 h injury**, R: **reperfusion**

ASK1 and VEGF may activate at the similar time point after cerebral ischemia.

2.3. *The protein levels of VEGF and phosphorylation-ASK1 in brain endothelial cells treated with ASK1 inhibitor*

To examine whether ASK1 directly affects the expression of VEGF in brain endothelial cells during OGD/R injury, we treated ASK1 inhibitor (NQDI-1) in bEND.3.cells before OGD/R injury. Fig. 3 shows that inhibition of ASK1 activity using NQDI-1 reduced the protein level of phosphorylation-ASK1 and VEGF compared to the OGD/R group at reperfusion 30 min after hypoxia injury (Fig. 3A and B). Our data suggest that ASK1 might play an important role in VEGF expression in brain endothelial cells after hypoxic injury. Furthermore, ASK1 may modulate the expression of VEGF at reperfusion early time point after OGD.

2.4. *Edema formation in MCAO mouse brain following ASK1-siRNA treatment*

To investigate whether ASK1 inhibition affects vascular permeability in animal brain, we measured brain edema at reperfusion 24 h after MCAO injury using TTC staining (Fig. 4A). White areas in brain are damaged brain areas due to ischemia (Fig. 4A). The graph shows the percentage of the ipsilateral hemisphere compared with the contralateral hemisphere both in the MCAO and si-ASK+MCAO groups (Fig. 4B). The percentage of brain edema in the MCAO group was >20% whereas the percentage of brain edema after si-ASK1 treatment was <10%. Brain edema (%) was significantly reduced in the si-ASK1+MCAO group compared with the MCAO group. Our results indicate that the inhibition of ASK1 reduced brain edema formation after ischemic brain injury. Considering this finding, the inhibition of ASK1 may be a useful strategy for reducing brain edema.

2.5. *Morphological alteration assessment using cresyl violet staining*

Cresyl violet staining was performed at reperfusion 24 h after MCAO injury to histologically assess the extent of ischemia-induced damage in the striatum and cortex (Fig. 5). In the NON group (without MCAO injury, without ASK1-siRNA treatment), intact cellular structure was observed in both the cortex and striatum. In the MCAO group, thin, small cell

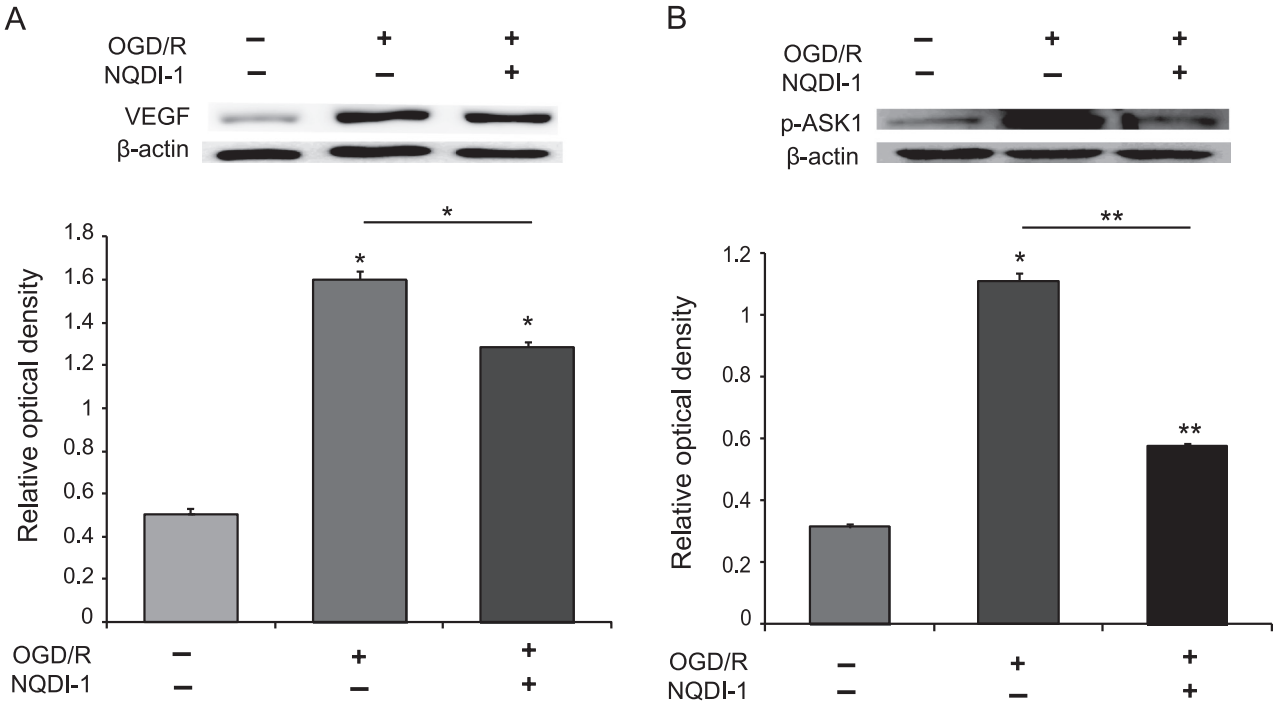

Fig. 3 – The protein level of VEGF and phosphorylation-ASK1 at reperfusion 30 min after OGD injury. (A)VEGF protein level was detected in bEND.3.cells at reperfusion 30 min after OGD 4hr injury. VEGF protein level was evidently increased in OGD/R group compared to the normal control group. However, VEGF was reduced in pretreatment of ASK1 inhibitor (NQDI-1) before OGD/R group compared with the OGD/R group. The protein level of β-actin was used as an internal control. (B) phosphorylation-ASK1 protein level was detected in bEND.3.cells at reperfusion 30 min after OGD 4hr injury. Phosphorylation-ASK1 protein level was strongly increased in OGD/R group compared to the normal control group (no OGD/R injury, no ASK1 inhibitor pretreatment). However, Phosphorylation-ASK1 was reduced in pretreatment of ASK1 inhibitor (NQDI-1) before OGD/R group compared with the OGD/R group. The protein level of β-actin was used as an internal control. Data were expressed as mean ± S.E.M, and were analyzed statistically using one-way ANOVA, followed by Bonferroni's post hoc. Statistical significance with the OGD/R group was determined by t-test. Each experiment included at least three replicates per condition. Differences were considered significant at $^{*}p<0.05$, $^{**}p<0.01$.
OGD/R: **reperfusion 30 min after OGD injury**, NQDI-1: **ASK1 inhibitor (NQDI-1) 3 h pretreatment before OGD injury**, p-ASK1: **phosphorylation- ASK1**

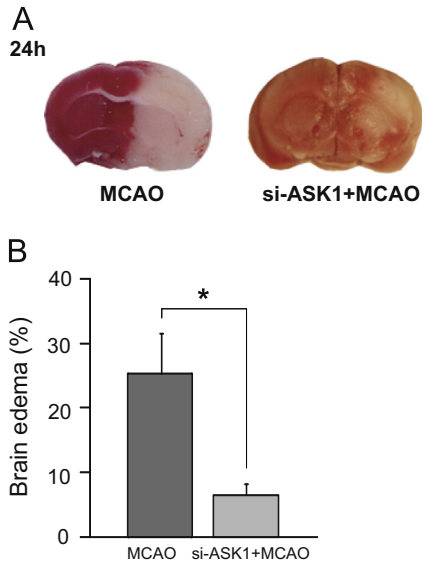

Fig. 4 – Measurement of edema formation in MCAO mouse brain at reperfusion 24 h after MCAO injury. (A) At reperfusion 24 h after MCAO injury, TTC staining showed that white areas were damaged by ischemic injury. White areas reduced in the si-ASK1 +MCAO group compared to the MCAO group. (B) Brain edema (%) was measured at reperfusion 24 h after MCAO injury. The quantitative graph shows that brain edema was significantly reduced in si-ASK1+MCAO group compared with the MCAO group. Data were expressed as mean ± S.E. M. Statistical significance with the MCAO group was determined by *t*-test. Differences were considered significant at $^{*}p<0.05$.
24 h MCAO group: **reperfusion 24hr after MCAO injury,** si-ASK1+MCAO group: **ASK1-siRNA treatment and reperfusion 24 h after MCAO injury.**

bodies were detected, and also damaged tissue was observed in the ischemic cortex and striatum. In the si-ASK1+MCAO group (ASK1-siRNA treatment and MCAO injury), damaged cells were reduced in number compared with the MCAO group, and we observed healthy round cells in the ischemic cortex and striatum. This data suggest that ASK1 inhibition may protect the brain tissue after cerebral ischemia.

2.6. *Immunohistochemistry to examine vascular permeability*

We performed immunohistochemistry using VEGF and AQP-1 antibody at reperfusion 24 h after MCAO injury to examine whether there were change of markers that affect vascular permeability (Figs. 6 and 7). We did not observe VEGF immunoreactivity in the cortex of the NON group (Fig. 6A). However, VEGF-positive cells were strongly expressed in the cortex in reperfusion 24hr after MCAO injury group. In addition, si-ASK1 transfected brain did not exhibit strong the expression of VEGF compared with 24 h MCAO group. In striatum, VEGF expression showed the same pattern as the cortex (Fig. 6B). In addition, the water channel molecule AQP-1 was detected in mouse brain cortex and striatum at 24 h after MCAO injury (Fig. 7). In the NON group, AQP-1 was not noticeably expressed. However, AQP-1 was evidently expressed in the cortex at reperfusion 24 h after MCAO injury group (Fig. 7A). In the si-ASK1+MCAO group, AQP-1 expression was lower in the cortex compared to reperfusion 24 h after MCAO injury group (Fig. 7A). AQP-1 immunoreactivity of the ischemic striatum was the same pattern as observed in the ischemic cortex (Fig. 7B). These data indicate that ASK1 affects the expression of VEGF and AQP-1 in ischemic brain and may be involved in vascular permeability and edema after ischemia.

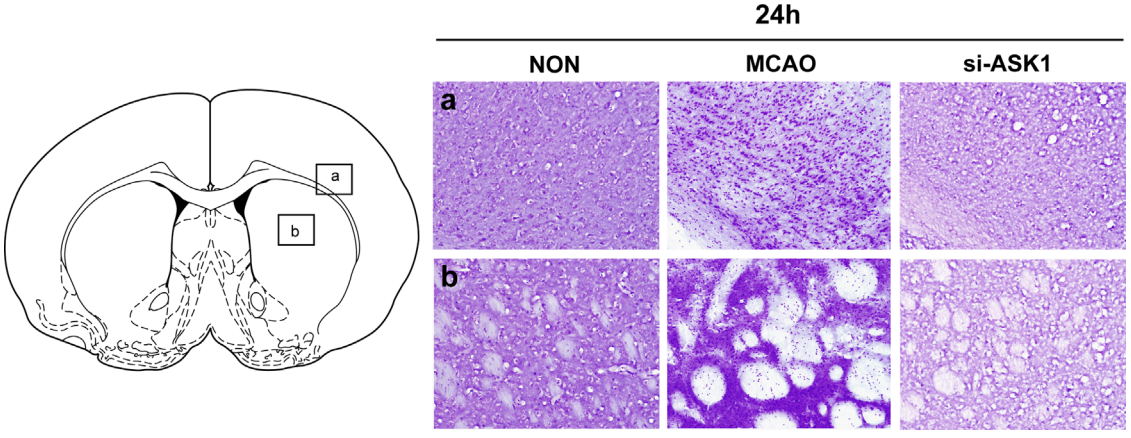

Fig. 5 – The histological assessment using cresyl violet staining after ischemic injury.
(A) Atlas of mouse brain mainly presents the corpus callosum, cerebral cortex (a) and striatum (b). (B) Cresyl violet staining indicated that severe cell loss was founded in the 24 h MCAO group whereas more healthy and round cell bodies in striatum and cortex were observed in ASK1-siRNA treatment before MCAO group.
a:**cortex,** b: **striatum,** NON: **normal control group,** MCAO: **reperfusion 24 h after MCAO injury,** si-ASK1: **ASK1-siRNA treatment and reperfusion 24 h after MCAO injury**

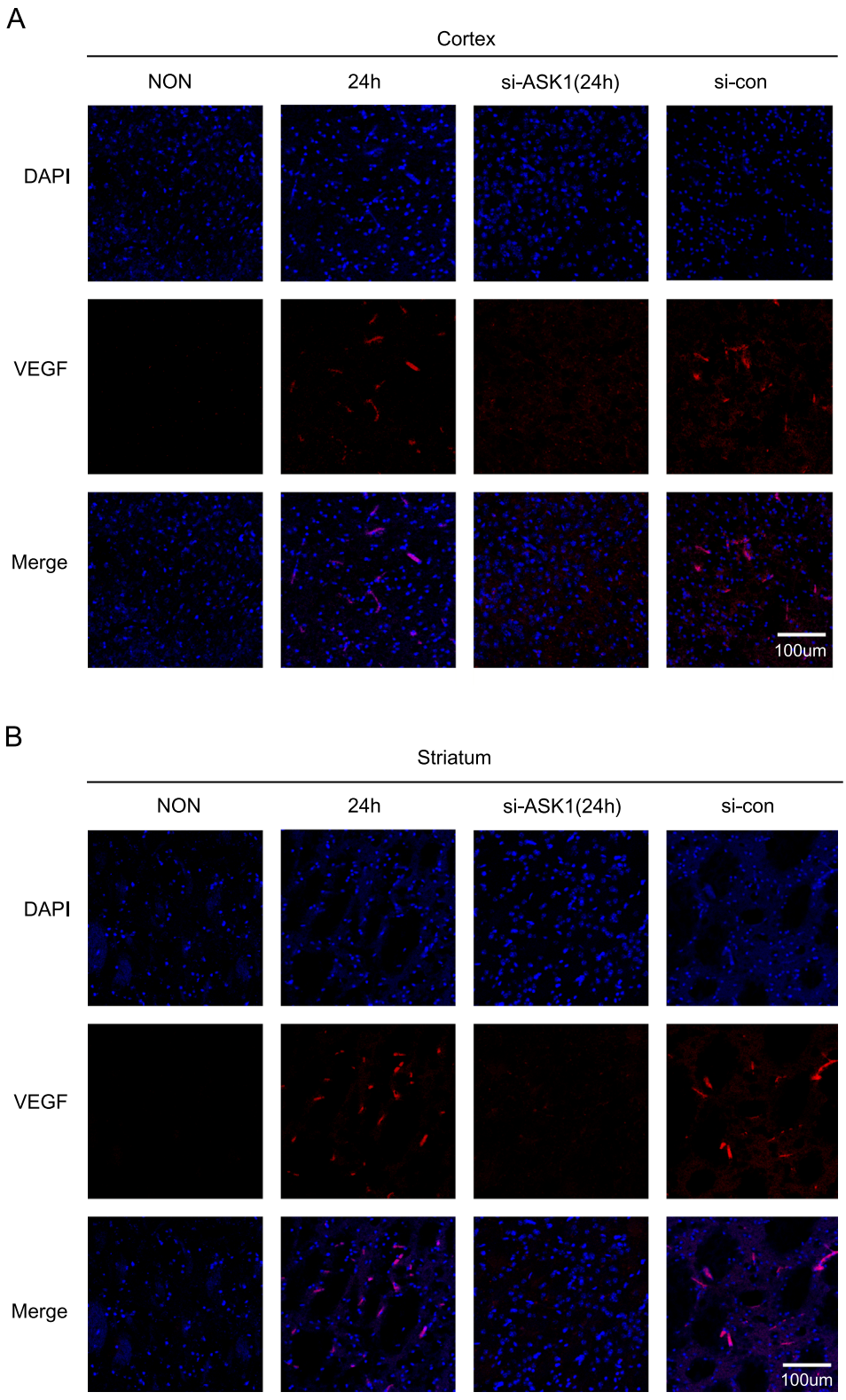

Fig. 6 – Immunochemical image for confirmation of reduced VEGF expression by ASK1-siRNA treatment.
(A) Immunochemical images showed that VEGF-positive cells (red) were densely expressed in MCAO mouse cortex. In si-ASK1 (24hr) group, VEGF expression was decreased in cortex, compared with the 24 h MCAO group. (B) In ischemic striatum (24 h group), many VEGF-positive cells were founded whereas in si-ASK1 (24hr) group, VEGF-positive cells were decreased in striatum owing to ASK1-siRNA treatment.
Scale bar=**100 μm,** VEGF**: red,** 4', 6-diamidino-2-phenylindole (DAPI)**: blue**
NON**: normal control group,** 24 h**: reperfusion 24hr after MCAO injury,** si-ASK1 (24 h)**: ASK1-siRNA treatment and reperfusion 24 h after MCAO injury,** si-con**: si-control treatment and reperfusion after MCAO injury.**

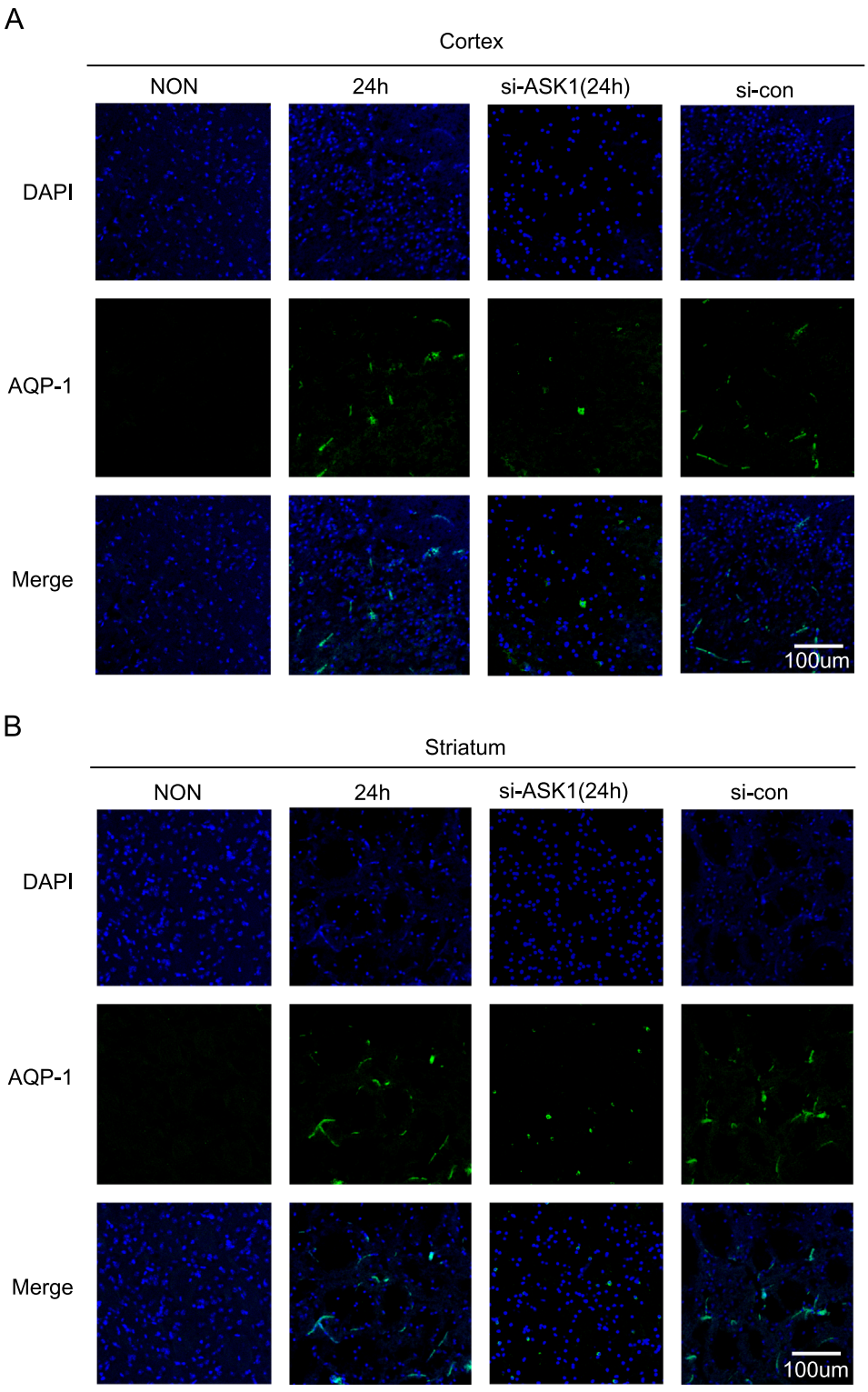

Fig. 7 – Immunochemical image for confirmation reduced AQP-1 expression by ASK1-siRNA treatment.
(A) Immunochemical images showed that AQP-1-positive cells (green) were densely expressed in MCAO mouse cortex. In si-ASK1 (24 h) group, AQP-1 expression was decreased in cortex, compared with the 24 h MCAO group. (B) In ischemic striatum (24 h group), many AQP-1-positive cells were founded whereas in si-ASK1 (24 h) group, AQP-1-positive cells were decreased in striatum owing to ASK1-siRNA treatment.
Scale bar=**100 μm,** AQP-1**: green,** 4', 6-diamidino-2-phenylindole (DAPI)**: blue**
NON**: normal control group,** 24 h**: reperfusion 24 h after MCAO injury,** si-ASK1 (24 h)**: ASK1-siRNA treatment and reperfusion 24 h after MCAO injury,** si-con**: si-control treatment and reperfusion after MCAO injury**

3. Discussion

Cerebral ischemia occurs following the occlusion of a cerebral artery by a thrombus and causes cell swelling due to cytotoxic edema and BBB disruption with vasogenic edema (Loreto and Reggio, 2010; Nakaji et al., 2006; Shibata et al., 2004). Vasogenic edema is directly linked to alteration of the BBB tight junctions with increasing permeability to many molecules (Ayata and Ropper, 2002; Heo et al., 2005). Several studies have demonstrated that edema is an important reason underlying clinical deterioration following ischemia and reperfusion (I/R) (Bounds et al., 1981; Davalos et al., 1999). The activation of ASK1 is regulated by the cellular redox state (Saitoh et al., 1998) and is associated with oxidative stress–induced BBB disruption (Toyama et al., 2014). In the present study, we suggested the role of ASK1 on vascular permeability and edema formation both in ischemia injured brain and in cultured brain endothelial cells under ischemia-induced oxidative stress. VEGF has been reported to exert protective effects on neurons (Mackenzie and Ruhrberg, 2012) and can enhance postischemic neurogenesis in brain (Sun et al., 2003; Wang et al., 2007; Wang et al., 2009). VEGF binds to its receptor (VEGFR) on endothelial cells and promotes many downstream signaling cascades, thus inducing vessel permeability and endothelial cell proliferation and migration (Gille et al., 2001; Keck et al., 1989; Waltenberger et al., 1994). Several studies have demonstrated that VEGF increases BBB permeability by stimulating the release of nitric oxide (Mayhan, 1999), and VEGF is involved in the degradation of the tight junction protein claudin-5, which contributes to a specific mechanism in BBB breakdown (Argaw et al., 2009). In addition, activation of the HIF-1α-VEGF pathway mediates the phosphorylation of tight junction proteins in response to hypoxic stress (Engelhardt et al., 2014). VEGF has been reported to reduce infarct size (Bellomo et al., 2003; Stowe et al., 2007; Stowe et al., 2008; Wang et al., 2005) and brain edema (Harrigan et al., 2002; Kimura et al., 2005; van Bruggen et al., 1999; Zhang et al., 2000) after cerebral ischemia. In transient MCAO mice, the relationship between VEGF and brain edema was shown in experiments with VEGFR-1 fusion protein (van Bruggen et al., 1999). Intravenous administration of VEGF to rats 1 h after MCAO was also demonstrated to reduce brain infarct size (Zhang et al., 2000). VEGF also induces the phosphorylation of ASK1 and c-Jun, which are related to JNK/SAPK signaling (Shen et al., 2012). A recent study suggested that oxidative stress-stimulated ASK1 activation leads to endothelial apoptosis, and VEGF suppresses endothelial apoptosis by inhibiting ASK1 activation (Nako et al., 2012). In the present study, we focused on the relationship between ASK1 and VEGF in hypoxia-induced brain endothelial cells and MCAO mouse brain to clarify the role of ASK1 in vascular permeability and edema formation. Our results suggest that ASK1 is associated with VEGF expression in brain endothelial cells at reperfusion early time point after hypoxia injury, and aggravates vascular permeability, and finally stimulates edema formation. Based on our results, ASK1 fast was activated in response to reperfusion condition after hypoxia injury and subsequently may stimulate vascular permeability in brain endothelial cells by modulating the expression of VEGF. AQP-1 is involved in brain water homeostasis (Arcienega et al., 2010) and is expressed in the apical membrane of the choroid plexus epithelium and in the lining of the cerebral ventricles (Oshio et al., 2005), where it plays an important role in cerebrospinal fluid (CSF) formation (Longatti et al., 2004; Nielsen et al., 1993). Recent studies have demonstrated that AQP-1 deletion in mice decreases the osmotic water permeability of the choroid plexus and lowers CSF production (Oshio et al., 2003; Oshio et al., 2005). Several studies have suggested that downregulation of AQP1 expression in the choroid plexus reduces brain edema formation (Kim et al., 2007), whereas its upregulation in endothelial cells leads to increased water permeability of the capillary walls and greater water entry to the brain (McCoy and Sontheimer, 2007). Others reported that AQP-1 expression changes in the ischemic stroke brain and is associated with edema formation (Badaut et al., 2007; Ribeiro Mde et al., 2006). In the present study, we found that ASK1 accelerated the activation of AQP-1 in the MCAO mouse brain. Considering our results, we suggest that the inhibition of ASK1 may attenuate increased osmotic water permeability following cerebral ischemia by inhibiting the activation of AQP-1 in ischemic brain. Taken together, our findings suggest that ASK1 may be activated at reperfusion early time point in cerebral ischemia and subsequently may be involved in the increase of VEGF and AQP-1 expression, ultimately resulting in edema formation. Thus, we conclude that the inhibition of ASK1 activation might be a target to treat clinical pathologies that occur after ischemic stroke.

4. Experimental procedure

4.1. *Cell culture*

Murine brain endothelial cells (bEnd.3 cells; ATCC, Manassas, VA, USA) were cultured in Dulbecco's modified Eagle's medium (DMEM, Hyclone Laboratories, Logan, UT, USA), supplemented with 10% (v/v) fetal bovine serum (FBS, Hyclone Laboratories, Logan, UT, USA) and 100 units/mL penicillin/streptomycin (Hyclone Laboratories, Logan, UT, USA), at 37 °C in a humidified atmosphere in the presence of 5% CO_2(Jung et al., 2013). bEND.3 cells were used in 13 passages.

4.2. *Oxygen–glucose deprivation and reperfusion (OGD/R)*

Confluent cells were transferred to an anaerobic chamber (Forma Scientific, Marietta, OH, USA) (O_2 tension, 0.1%) and washed three times with phosphate-buffered saline (PBS). Then, the culture medium was replaced with de-oxygenated, glucose-free balanced salt solution, and cells were incubated for 4 h in the anaerobic chamber. Following oxygen–glucose deprivation (OGD) injury, cells were incubated for 30 min, 1 h, 3 h under normal growth conditions, respectively (Yang et al., 2007). bEND.3 cells were pretreated with 600 nM ASK1 inhibitor (NQDI-1, Tocris Bioscience, Bristol, UK) to inhibit ASK1 activation 3 h before hypoxia stress.

4.3. *Animal model*

Male C57BL/6 mice (Orient, GyeongGi-Do, Korea; 8- to 12-week old) were subjected to transient focal cerebral ischemia by intraluminal middle cerebral artery blockade with a nylon suture, as previously described (Unterberg et al.,

2004). After 60 min of middle cerebral artery occlusion (MCAO), blood flow was restored by withdrawing the suture, and regional cerebral blood flow was monitored using a laser Doppler flow meter (Transonic Systems, Inc., Ithaca, NY, USA). All animal procedures and experiments were performed in accordance with the Guide to the Care and Use of Laboratory Animals and were approved by the Association for Assessment and Accreditation of Laboratory Animal Care.

4.4. Preparation of ASK1 targeting siRNA

An si-RNA targeting ASK1 (Ambion, Austin, TX, USA; sense: GCUGGUAAUUUAUACACuGtt, antisense: CAGUGUAUAAAUUACGAGCtt, concentration: 5 μM) was used in this study. A mixture of siPORTNeoFX (Ambion, Austin, TX, USA) and ASK1-siRNA was injected into the lateral ventricles of the mouse brain (mediolateral 1.0 mm; anteroposterior 0.2 mm; dorsoventral 3.1 mm) with an osmotic pump (Alzet, Cupertino, CA, USA) 3 days before inducing MCAO injury. ASK1-siRNA was infused at a rate of 1 μl/h. Scrambled si-RNA as a control was infused in the same way.

4.5. Microarray analysis

The mouse brains were homogenized with TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's recommendations 8 h after occlusion. In addition, Agilent's Low RNA Input Linear Amplification kit (Agilent Technology, Santa Clara, CA, USA) was used, and double-stranded DNA was transcribed by adding the transcription master mix (4× transcription buffer, 0.1 M DTT, NTP mix, 50% PEG, RNase-out, inorganic pyrophosphate, T7-RNA polymerase and cyanine 3/5-CTP) to the double-stranded DNA reaction samples and incubating at 40 °C for 2 h. After testing the efficiency of labeling, the fragmented cRNA was pipetted onto a Whole Human Genome Microarray Kit (4 × 44 K, Agilent Technology, Santa Clara, CA, USA), and the hybridized microarrays were washed following the manufacturer's protocol. Using Agilent's DNA microarray scanner, the hybridized images were scanned and quantified using Feature Extraction (Agilent Technology, Santa Clara, CA, USA) and GeneSpringGX7.3 (Agilent Technology, Santa Clara, CA, USA) software, all data were normalized, and genes of interest were selected based on the fold change.

4.6. Western blot analysis

After pre-treatment, OGD injury, and restoration, cells were washed rapidly with ice-cold PBS, scraped, and collected. Cell pellets were lysed with ice-cold RIPA buffer (Sigma-Aldrich, St. Louis, MO, USA). The lysates were centrifuged at 13,200 rpm for 1 h at 4 °C to produce whole-cell extracts. Protein content was quantified using the BCA method (Pierce, Rockford, IL, USA). Protein (20 μg) was separated on a 10% SDS–polyacrylamide (PAGE) gel and transferred onto a polyvinylidene difluoride (PVDF) membrane. After blocking with 5% bovine serum albumin, prepared in Tris-buffered saline/Tween (TBS-T; 20 nM Tris [pH 7.2], 150 mM NaCl, and 0.1% Tween 20), for 1 h at room temperature (RT), immunoblots were incubated overnight at 4 °C with primary antibodies that specifically detect ASK1 (1:500, Santa Cruz Biotechnology, Santa Cruz, CA, USA), phosphorylation-ASK1 (1:500, Santa Cruz Biotechnology, Santa Cruz, CA, USA),VEGF (1:1000, Millipore, Billerica, MA, USA), or β-actin (1:2000, Cell Signaling Technology, Danvers, MA, USA). Next, blots were incubated with HRP-linked anti-mouse and -rabbit IgG antibodies purchased from Abcam (Cambridge, UK) for 1 h at RT. Enhanced chemiluminescence was performed by ECL (Pierce) (Jung et al., 2013).

4.7. Evaluation of brain edema

For the evaluation of brain edema, mice were sacrificed at reperfusion 24 h after MCAO injury. Isolated brains were incubated with 2% 2, 3, 5-triphenyltetraxolium chloride (TTC) (Sigma-Aldrich, St. Louis, MO, USA) at 37 °C for 10 min in the dark in a drying oven. The ipsilateral and contralateral hemispheres were used to calculate the percentage of brain edema (Mohammadi et al., 2012).

$$\text{Brain edema (\%)} = \frac{(\text{volume of ipsilateral hemisphere} - \text{volume of contralateral hemisphere})}{\text{volume of contralateral hemisphere}} \text{ X } 100$$

4.8. Cresyl violet staining

At reperfusion 24 h after MCAO injury, mice were sacrificed and brains were fixed in 3.7% formaldehyde and quickly frozen. Tissues were sectioned coronally at 20 μm thickness and sequentially dipped into xylene 5 min, 100% alcohol 5 min, 95% alcohol 5 min, and 70% alcohol 5 min. Samples were stained with cresyl violet (Sigma-Aldrich, St. Louis, MO, USA) for 3 min. After the staining, slides were reacted with 70% alcohol 5 min, 95% alcohol 5 min, 100% alcohol 5 min, and xylene 5 min. After these processes, sections were observed under a microscope equipped with a digital camera (Olympus, Tokyo, Japan).

4.9. Immunohistochemistry

Five-micrometer-thick frozen brain sections were cut onto clean glass slides (Thermo Scientific, Waltham, MA, USA), air-dried, and fixed in cold acetone for 10 min at –20 °C. The slides were first washed in Tris-buffered saline (TBS) and then incubated with 0.3% H_2O_2 in methanol to quench endogenous peroxidase activity. Followed by a series of washes (three times with distilled water), the sections were blocked with 10% normal rabbit serum. Frozen brain sections (20 μm) were fixed in ice-cold acetone for 20 min. To block nonspecific labeling, sections were incubated in 5% bovine serum albumin (BSA) (Sigma-Aldrich, St. Louis, MO, USA) diluted in PBS for 30 min before addition of primary and secondary antibodies. Primary antibodies for VEGF (1:50, Millipore, Billerica, MA, USA), AQP-1 (1:50, Abcam, Cambridge, MA, USA) were applied to the samples for 24 h at 4 °C, followed by a 90 min incubation with appropriate florescence secondary antibody (1:100, Invitrogen, Carlsbad, CA, USA) and three washes in PBS for 10 min each. After three washes in 0.1% PBS with Tween-20 (PBST), the sections were incubated with rhodamine-conjugated

sheep anti-rabbit or FITC-conjugated sheep anti-mouse secondary antibody that was diluted to 1:200 with 5% BSA fraction V in 0.1% PBST for 2 h in the dark at RT. After three washes in PBS, all sections were incubated with 1 μg/mL of 4',6-diamidino-2-phenylindole (Sigma-Aldrich, St. Louis, MO, USA) and 2 μg/mL of propidium iodide (Sigma-Aldrich, St. Louis, MO, USA) for a counter staining. Tissues were then visualized under a confocal microscope (Zeiss LSM 700, Carl Zeiss, Oberkochen, Germany).

4.10. *Statistical analysis*

Statistical analyses were carried out using SPSS 18.0 software (IBM Corp., Armonk, NY, USA). All data are expressed as mean ± S.E.M. Significant intergroup differences were determined by one-way analysis of variance followed by Bonferroni *post hoc* multiple comparison test. Statistical significance with the OGD/R or MCAO group was determined by *t*-test. Each experiment included at least three replicates per condition. Differences were considered significant at $^{*}p<0.05$, $^{**}p<0.01$.

Conflicts of interest

The authors declare no conflict of interest regarding the publication of this paper.

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