Expression Changes of Neuroprotective Signaling Molecules in the Mouse Cortex Following Permanent Middle Cerebral Artery Occlusion

-Neuroprotective Signaling In pMCAO-

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Key Words: MCAO, NRF, HO-1, BDNF, AKT

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Abstract

Oxidative stress and altered intracellular signaling contribute to ischemic brain injury; however, molecular alterations during the subacute phase following permanent cerebral ischemia remain incompletely understood. In this study, nine-week-old male mice underwent permanent middle cerebral artery occlusion using the intraluminal filament method, and ipsilateral cortical tissue was collected three days after ischemia. Western blot analysis was performed to evaluate nuclear factor erythroid 2-related factor 2, heme oxygenase-1, brain-derived neurotrophic factor, and the activation of protein kinase B and extracellular signal-regulated kinase. Nuclear factor erythroid 2-related factor 2 and heme oxygenase-1 expression levels were significantly decreased following permanent cerebral ischemia, whereas brain-derived neurotrophic factor expression was significantly increased. Protein kinase B activation, assessed by the ratio of its phosphorylated to total form, was significantly reduced, whereas extracellular signal-regulated kinase activation showed no significant difference between the control and ischemia groups. These findings demonstrate distinct alterations in antioxidant defense, neurotrophic responses, and intracellular survival signaling in the cerebral cortex three days after permanent cerebral ischemia and provide insight into molecular responses associated with the subacute stage of ischemic injury.

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Introduction

Stroke is one of the leading causes of mortality and long-term disability worldwide (1). Cerebral ischemia initiates a complex cascade of pathological events, including energy depletion, excitotoxicity, oxidative stress, inflammation, and neuronal apoptosis (2, 3). Among these pathological mechanisms, oxidative stress plays a central role by promoting excessive production of reactive oxygen species (ROS), resulting in cellular dysfunction and neuronal death (4).

Cells possess endogenous antioxidant defense systems to counteract oxidative injury. Nuclear factor erythroid 2-related factor 2 (NRF2) is a master transcription factor that regulates antioxidant responses by inducing various cytoprotective genes, including heme oxygenase-1 (HO-1)(5, 6). Activation of the NRF2/HO-1 pathway has been shown to reduce oxidative damage and improve neuronal survival in experimental stroke models (7). Conversely, impaired NRF2 signaling may exacerbate ischemic brain injury.

Neuronal survival following cerebral ischemia is also regulated by intracellular signaling pathways. The phosphatidylinositol 3-kinase (PI3K)/protein kinase B (AKT) pathway promotes cell survival and inhibits apoptosis(8), whereas extracellular signal-regulated kinase (ERK) signaling participates in neuronal plasticity, differentiation, and stress responses(9). Alterations in these pathways influence neuronal fate after ischemic injury and may contribute to functional recovery.

Brain-derived neurotrophic factor (BDNF) is another key mediator involved in neuronal survival, synaptic plasticity, and post-ischemic repair (10, 11). Experimental studies have demonstrated that BDNF expression may increase following ischemic injury as an endogenous compensatory response(12). However, the relationship between BDNF expression and antioxidant or intracellular survival signaling during the subacute phase after permanent cerebral ischemia remains unclear.

Therefore, the present study investigated alterations in antioxidant defense molecules (NRF2 and HO-1), neurotrophic signaling (BDNF), and intracellular survival pathways (AKT and ERK) in the cerebral cortex three days after permanent middle cerebral artery occlusion in mice. Understanding these molecular changes may provide insight into endogenous neuroprotective responses during the subacute stage of ischemic stroke.

Material and Methods

Animal Surgery

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All animal procedures were performed in accordance with the guidelines of the Institutional Animal Care and Use Committee of Chosun University. Nine-week-old male Institute of Cancer Research (ICR) mice were obtained from Damul Laboratory Animals (Daejeon, Republic of Korea). Only male mice were used in this study to minimize potential biological variability associated with the estrous cycle and to maintain experimental consistency. Mice were divided into a naïve control group and a permanent middle cerebral artery occlusion (pMCAO) group. Control mice did not undergo anesthesia or surgical procedures and were used as the baseline control for protein expression analyses. Mice were anesthetized with 2.0% sevoflurane throughout the surgical procedure. Core body temperature was maintained at 37 ± 0.5°C using a rectal temperature probe connected to a temperature controller (JD-OT-06DT, JEUNG-DO BIO, Seoul, Republic of Korea).

A midline cervical incision was made to expose the left common carotid artery (CCA), which was carefully separated from the vagus nerve. The external carotid artery (ECA) and CCA were ligated using 4-0 silk sutures. A small arteriotomy was created in the CCA, and a silicone-coated 6-0 nylon monofilament (ETHICON, Raritan, NJ, USA) was inserted through the CCA and advanced into the internal carotid artery until the origin of the middle cerebral artery was occluded. Cerebral blood flow (CBF) was continuously monitored during surgery using a laser Doppler flowmeter (Periflux 6000; Perimed, Stockholm, Sweden). Successful induction of ischemia was defined as a reduction in CBF of at least 80% from baseline. All animals included in the pMCAO group met this predefined criterion. No animals were excluded after surgery, and no mortality occurred before the planned experimental endpoint (3 days after pMCAO). The present study focused on molecular analysis by western blotting; therefore, histological evaluation of infarct volume was not performed.

Animals were allocated to the experimental groups according to the predetermined study design, and formal randomization was not performed. Western blot images were quantified using ImageJ software by a researcher who was not involved in the surgical procedures. Formal blinding to group assignment was not implemented.

To establish a pMCAO model, the filament was left in place without withdrawal throughout the experimental period. After surgery, the cervical incision was closed, and the animals were allowed to recover with free access to food and water. Three days after pMCAO induction, mice were deeply anesthetized and sacrificed. The ipsilateral cerebral cortex was rapidly dissected, immediately frozen in liquid nitrogen, and stored at −80°C until western blot analysis.

Western blotting

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All mice (control group, n = 5; pMCAO group, n = 9) were deeply anesthetized with sevoflurane (1.0–2.0% end-tidal concentration), and the ipsilateral cerebral cortex was rapidly isolated. Tissue samples were homogenized in radioimmunoprecipitation assay (RIPA) buffer supplemented with one tablet of protease inhibitor cocktail (11836153001, Roche, Basel, Switzerland) per 10 mL of lysis buffer at a volume of five times the tissue weight. The homogenates were centrifuged, and the supernatants were collected for protein extraction. Total protein concentrations were determined using the Bradford protein assay.

Equal amounts of protein were denatured in sodium dodecyl sulfate (SDS) sample buffer, separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE), and transferred onto nitrocellulose membranes (10600001, GE Healthcare, Piscataway, NJ, USA). The membranes were incubated with primary antibodies against NRF2 (1:1000, NBP1-32822, Novus Biologicals, USA), HO-1 (1:500, sc-390991, Santa Cruz Biotechnology, USA), BDNF (1:1000, ab108319, Abcam, UK), phosphorylated AKT (pAKT, 1:1000, #9271, Cell Signaling Technology, USA), total AKT (AKT, 1:1000, #9272, Cell Signaling Technology, USA), phosphorylated ERK (pERK, 1:1000, #9101, Cell Signaling Technology, USA), total ERK (ERK, 1:1000, #9102, Cell Signaling Technology, USA), and β-actin (1:2000, 66009-1-Ig, Proteintech, USA). Protein bands were quantified using ImageJ software (National Institutes of Health, Bethesda, MD, USA). β-Actin served as the internal loading control, and protein expression levels were normalized to the corresponding β-actin signals.

Statistical analysis

Statistical analyses were performed using GraphPad Prism 11 (GraphPad Software, San Diego, CA, USA). Data are presented as mean ± SEM. Normality of the data distribution was assessed using the Shapiro–Wilk test, and all datasets showed no significant deviation from normality ($P > 0.05$). Comparisons between the control and MCAO groups were performed using an unpaired Student's t-test. A P-value < 0.05 was considered statistically significant.

Resluts

Decreased expression of NRF2 and HO-1 following permanent MCAO

To evaluate changes in antioxidant defense following permanent cerebral ischemia, the protein expression levels of NRF2 and HO-1 were analyzed by western blotting. As shown in Figure 1, the expression of NRF2 was significantly decreased in the pMCAO group compared with the control group. Similarly, HO-1 protein expression was also significantly reduced following pMCAO, indicating suppression of the endogenous

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antioxidant defense system in the ischemic cortex.

Increased BDNF expression in the ischemic cortex

The expression of BDNF was examined to evaluate neurotrophic responses after permanent cerebral ischemia. Western blot analysis demonstrated that BDNF protein expression was significantly increased in the pMCAO group compared with the control group (Figure 1).

Decreased AKT activation after pMCAO

To investigate changes in cell survival signaling, the activation level of AKT was evaluated. The ratio of pAKT to AKT was significantly lower in the pMCAO group than in the control group (Figure 2), suggesting reduced activation of the AKT signaling pathway following permanent cerebral ischemia.

ERK activation was not significantly altered after permanent MCAO

The activation status of ERK was also assessed by western blot analysis. No significant difference in the pERK/ERK ratio was observed between the control and pMCAO groups (Figure 3), indicating that ERK signaling remained unchanged at three days after permanent cerebral ischemia.

Discussion

Most previous experimental studies have focused on transient middle cerebral artery occlusion (tMCAO) models or the acute phase of ischemia–reperfusion injury. In contrast, the present study investigated molecular alterations in the cerebral cortex during the subacute phase following pMCAO. We demonstrated that pMCAO significantly reduced the expression of NRF2 and HO-1 and suppressed AKT activation, whereas BDNF expression was significantly increased. In contrast, ERK activation was not significantly altered. These findings suggest that prolonged cerebral ischemia impairs endogenous antioxidant defense and cell survival signaling while simultaneously eliciting a compensatory neurotrophic response. Together, these findings provide insight into the molecular responses that occur during the subacute stage of permanent cerebral ischemia and may contribute to a better understanding of ischemic stroke pathophysiology.

NRF2 is a master regulator of the endogenous antioxidant defense system and plays a pivotal role in protecting neurons against oxidative stress following cerebral ischemia (13). Upon activation, NRF2 translocates to the nucleus and induces the expression of several antioxidant and cytoprotective genes, including HO-1, thereby limiting ROS-mediated neuronal injury(14). Numerous experimental studies have demonstrated that activation

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of the NRF2/HO-1 pathway attenuates oxidative damage and improves neurological outcomes after ischemic stroke (15, 16).

In the present study, both NRF2 and HO-1 protein expression were significantly decreased in the cerebral cortex three days after pMCAO. These findings suggest that prolonged cerebral ischemia impairs endogenous antioxidant defense during the subacute stage of ischemic injury. Unlike many previous studies, which primarily investigated tMCAO models or the acute phase following reperfusion, the present study evaluated molecular changes three days after permanent arterial occlusion. Persistent cerebral hypoperfusion may overwhelm intrinsic antioxidant defense mechanisms, resulting in suppression of the NRF2/HO-1 pathway despite ongoing oxidative stress (17, 18).

BDNF is a key neurotrophic factor that regulates neuronal survival, synaptic plasticity, and functional recovery following cerebral ischemia (19, 20). Previous studies have demonstrated that endogenous BDNF expression is upregulated after ischemic brain injury and contributes to neuronal remodeling and neuroplasticity during the recovery process(21, 22). Rather than directly indicating successful neuroprotection, increased BDNF expression is generally regarded as an intrinsic compensatory response to ischemic injury that aims to promote neuronal survival and repair.

Consistent with these observations, the present study demonstrated a significant increase in BDNF protein expression in the cerebral cortex three days after pMCAO. Previous studies using tMCAO models have reported that endogenous BDNF expression increases predominantly during the recovery phase (23)., whereas our study demonstrated elevated BDNF expression at day 3 after pMCAO. This difference may reflect distinct molecular responses associated with persistent ischemia in the absence of reperfusion. Under conditions of sustained ischemic stress, endogenous BDNF expression may be enhanced as an adaptive attempt to counteract ongoing neuronal injury and maintain tissue homeostasis (24). However, despite the increased expression of BDNF, AKT phosphorylation was significantly reduced in the present study, indicating that increased BDNF expression occurred despite impaired activation of intracellular survival signaling during prolonged cerebral ischemia. These findings indicate that although neurotrophic responses are activated during the subacute stage of permanent ischemia, they may not adequately overcome persistent oxidative stress and irreversible ischemic damage.

The pPI3K/AKT signaling pathway is one of the principal intracellular pathways regulating neuronal survival,

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apoptosis, and cellular responses to ischemic injury. Activation of AKT through phosphorylation promotes cell survival by suppressing apoptotic signaling and enhancing resistance to oxidative stress (25, 26). Numerous experimental studies have demonstrated that increased AKT activation is associated with reduced neuronal injury and improved functional recovery following cerebral ischemia(26).

In the present study, the pAKT/AKT ratio was significantly decreased in the cerebral cortex three days after pMCAO, indicating impaired activation of this pro-survival signaling pathway. Although increased BDNF expression and reduced AKT activation were observed simultaneously, the present study was not designed to determine a mechanistic relationship between these molecular alterations (27). Persistent ischemia and continuous metabolic stress in the absence of reperfusion may impair intracellular signal transduction despite increased neurotrophic factor expression. Consequently, the reduction in AKT activation observed in the present study may indicate insufficient activation of endogenous survival pathways during prolonged cerebral ischemia, thereby contributing to progressive neuronal injury. In contrast, ERK activation was not significantly altered three days after pMCAO. The ERK signaling pathway has been reported to exert both neuroprotective and detrimental effects following cerebral ischemia depending on the severity, duration, and experimental conditions of injury (28, 29). The absence of significant changes in ERK activation in the present study suggests that prolonged permanent ischemia may differentially regulate intracellular survival pathways, with AKT appearing to be more susceptible than ERK under the present experimental conditions.

The present study has several limitations. First, the control group consisted of naïve mice rather than sham-operated animals, and histological confirmation of infarction and neurobehavioral assessments were not performed because this study was designed as an exploratory molecular investigation. In addition, because western blot analyses were performed using whole cortical tissue, the present study could not determine whether the observed molecular alterations were attributable to specific cellular populations or histopathological changes within the ischemic cortex. Therefore, the present findings should be interpreted as molecular alterations associated with permanent cerebral ischemia rather than direct evidence of functional neuroprotection or recovery. Second, molecular analyses were performed only at a single post-ischemic time point (3 days after pMCAO), precluding evaluation of the temporal progression of these signaling pathways. Finally, only male mice were included in this study, and therefore the present findings may not fully represent sex-specific molecular responses to cerebral ischemia. Future studies incorporating sham-operated controls, histological and behavioral assessments, multiple post-ischemic time points, and both sexes will be necessary to further validate

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the biological significance of these molecular alterations.

Conclusion

In conclusion, the present study demonstrated that permanent cerebral ischemia induced distinct alterations in intracellular signaling pathways associated with oxidative stress, neurotrophic responses, and neuronal survival. Persistent ischemia significantly reduced the expression of NRF2 and HO-1, indicating impaired endogenous antioxidant defense, while BDNF expression was increased, suggesting a compensatory neurotrophic response. Despite this increase in BDNF, AKT activation was significantly decreased, implying insufficient activation of endogenous survival signaling during prolonged ischemia, whereas ERK activation remained unchanged under the present experimental conditions. Collectively, these findings suggest that persistent cerebral ischemia differentially regulates intracellular signaling pathways and provide insight into molecular responses observed during the subacute stage of permanent ischemia. Further studies incorporating multiple post-ischemic time points, together with histological and neurobehavioral assessments, will be required to determine the temporal progression and functional significance of these molecular alterations.

Conflicts of Interest: The authors declare no competing interests

Acknowledgements: This study was supported by research funds from Chosun University (2025).

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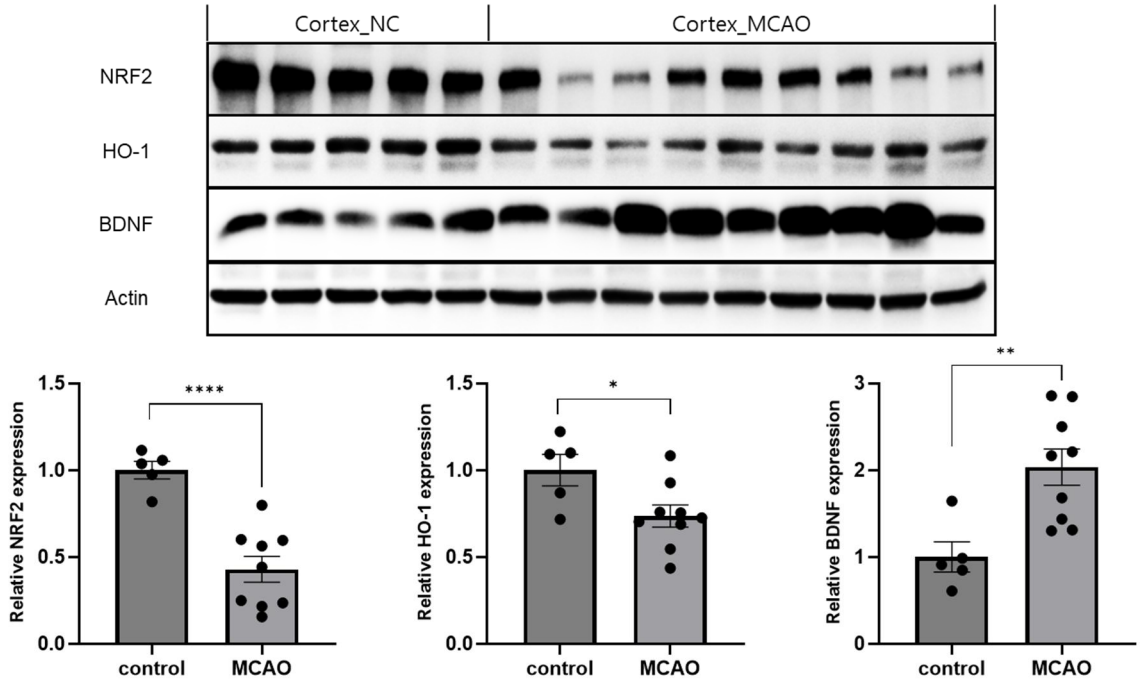


Figure 1 Changes in NRF2, HO-1, and BDNF expression following permanent MCAO. Representative western blot images and quantitative analyses of NRF2, HO-1, and BDNF expression in the cerebral cortex of control and permanent middle cerebral artery occlusion (MCAO) mice. Protein expression was normalized to β-actin and expressed relative to the mean value of the control group, which was set to 1.0. NRF2 and HO-1 expression levels were significantly decreased in the MCAO group, whereas BDNF expression was significantly increased. Data are presented as mean ± SEM (control, n = 5; MCAO, n = 9). *P < 0.05, **P < 0.01, ****P < 0.0001 versus the control group.

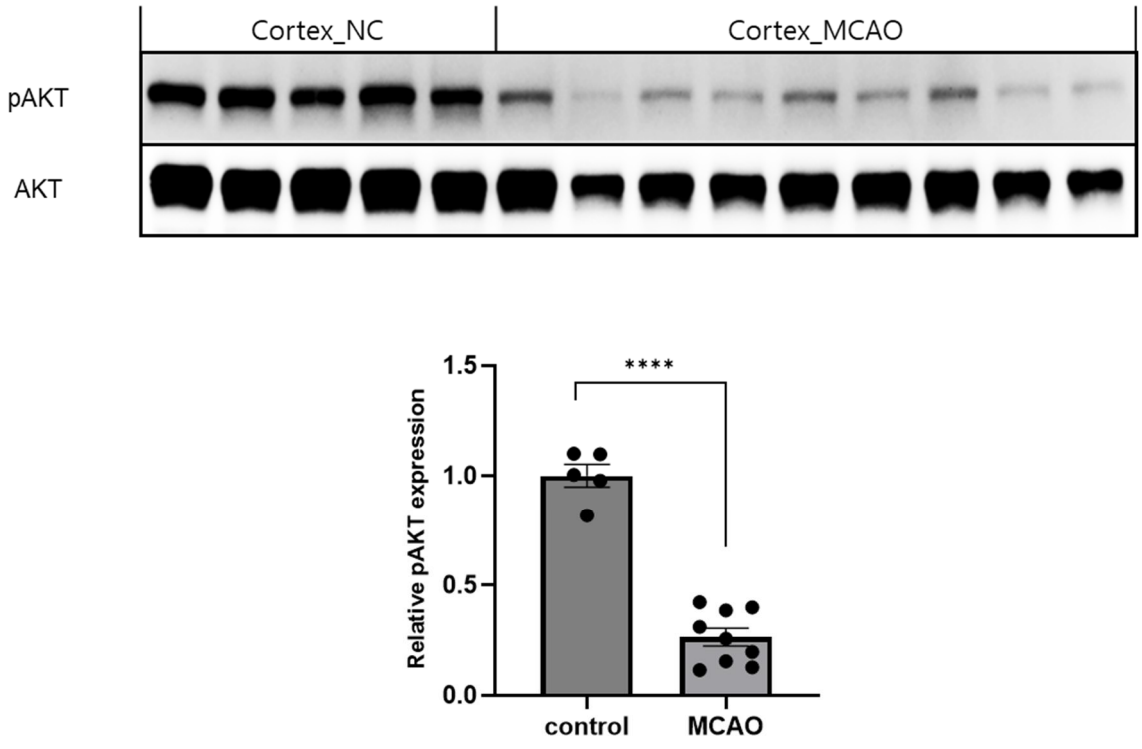


Figure 2. Reduced AKT activation following permanent MCAO. Representative western blot images and quantitative analysis of AKT activation in the cerebral cortex of control and permanent MCAO mice. AKT

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activation was assessed using the phospho-AKT (pAKT)/total AKT ratio. AKT activation was significantly reduced in the MCAO group compared with the control group. Data are presented as mean ± SEM (control, n = 5; MCAO, n = 9). ****P < 0.0001 versus the control group.

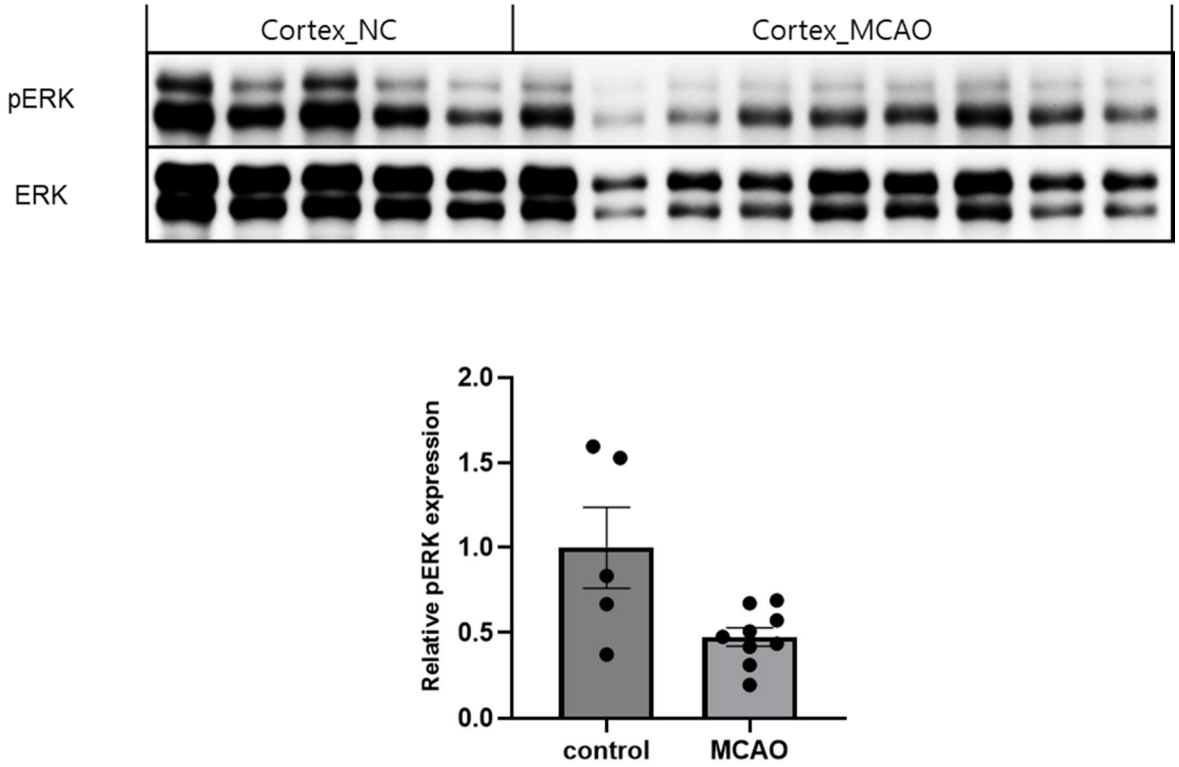


Figure 3. ERK activation following permanent MCAO.

Representative western blot images and quantitative analysis of ERK activation in the cerebral cortex of control and permanent MCAO mice. ERK activation was assessed using the phospho-ERK (pERK)/total ERK ratio. No statistically significant difference in ERK activation was observed between the control and MCAO groups. Data are presented as mean ± SEM (control, n = 5; MCAO, n = 9).

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