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# Estradiol alleviates the ischemic brain injury-induced decrease of neuronal calcium sensor protein hippocalcin

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## HIGHLIGHTS

- Estradiol plays a neuroprotective role against neuronal cell injury.
- Estradiol prevents brain injury-induced decrease of in hippocalcin levels.
- Estradiol attenuates the glutamate treatment-induced decrease in hippocalcin levels.

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## ABSTRACT

Estradiol has protective and reparative effects in neurodegenerative diseases. Hippocalcin is a neuronal calcium-sensor protein that acts as a calcium buffer to regulate the intracellular concentration of $Ca^{2+}$. This study was investigated to elucidate whether estradiol regulates hippocalcin expression in a focal cerebral ischemia model and glutamate-treated neuronal cells. An ovariectomy was performed in adult female rats, and vehicle or estradiol was administered before middle cerebral artery occlusion (MCAO). Cerebral cortex tissues were collected at 24 h after MCAO. A proteomic approach revealed that hippocalcin expression decreased in vehicle-treated animals with combined MCAO, while estradiol treatment attenuated this decrease. Reverse transcription-PCR and Western blot analyses also showed that estradiol administration prevented the MCAO injury-induced decrease in hippocalcin expression. In cultured hippocampal cells, glutamate exposure increased the intracellular $Ca^{2+}$ concentration, which was rescued by the presence of estradiol. Moreover, glutamate toxicity decreased hippocalcin expression, whereas estradiol attenuated this decrease. Together, these findings suggest that estradiol has a neuroprotective function by regulating hippocalcin expression and intracellular $Ca^{2+}$ levels in ischemic brain injury.



Estradiol is a representative female sexual steroid hormone. In addition to its reproductive function, estradiol plays a neuroprotective role against neuronal cells injury [26,28]. Estradiol prevents neuronal cells death in response to oxidative stress and alleviates brain lesions in focal cerebral ischemia [5,7]. Clinical studies have demonstrated that estradiol reduces the progression of Alzheimer's disease and the risk of stroke [7,19]. Consistent with this, the risk of neurological diseases increases in postmenopausal women compared to age-matched men [3,22]. However, premenopausal women suffer less from neurodegenerative diseases than adult men [22]. Moreover, estradiol replacement therapy reduces the mortality of stroke-related deaths [23].

Intracellular $Ca^{2+}$ is involved in neuronal cells growth, neuronal transmission, and synaptic plasticity [2]. However, an excessive increase in intracellular $Ca^{2+}$ activates the caspase cascade, leading to cell death and neuronal disorder [9]. Ischemic brain injury increases intracellular $Ca^{2+}$ levels and leads to apoptotic and necrotic cell death [25]. Hippocalcin is a calcium-binding protein that is expressed mainly in the brain. It belongs to the neuronal calcium-sensor protein family and acts as a calcium buffer because it binds to $Ca^{2+}$ that is released in the cytoplasm [10,21]. Thus, regulation of hippocalcin expression is considered important for intracellular $Ca^{2+}$ homeostasis. Moreover, it has been reported that estradiol reduces the increase in intracellular $Ca^{2+}$ concentration that occurs following neuronal injury [14]. Although previous studies have demonstrated that estradiol regulates $Ca^{2+}$concentration, it is unclear whether hippocalcin is regulated by estradiol in ischemic brain injury. A proteomics technique was used to identify various proteins that are differentially expressed following

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estradiol treatment during ischemic brain injury. Among these identified proteins, we focused on change of hippocalcin. In this study, we investigated whether estradiol regulates hippocalcin expression in a middle cerebral artery occlusion (MCAO) animal model and glutamate-exposed neuronal cells.

Female Sprague-Dawley rats (220–230 g, $n = 60$) were purchased from Samtako Co. (Animal Breeding Center, Korea) and maintained under controlled temperature and lighting (12 h/12 h light/dark cycle) with free access to food and water. Animals were divided randomly into the following four groups: vehicle + sham, estradiol + sham, vehicle + MCAO, and estradiol + MCAO groups ($n = 15$ per group). Animals were subjected to an ovariectomy and were implanted with a silastic capsule containing sesame oil (Sigma, St. Louis, MO, USA. vehicle) or 17β-estradiol (180 μg/ml, Sigma). This capsule of 17β-estradiol consistently produces estradiol levels equivalent to circulating physiological levels [5].

Animals were anesthetized with sodium pentobarbital (100 mg/kg) and MCAO was performed as described previously [15]. Briefly, the bifurcation of the right common carotid artery was exposed through a midline incision. The internal carotid artery and external carotid artery were dissected from the adjacent tissues. A 4/0 monofilament nylon with its tip rounded by heat was gently inserted from the external carotid artery into the internal carotid artery, and advanced up to the origin of the middle cerebral artery. Sham-operated animals were subjected to the same surgical process without arterial blockade.

Hematoxylin and eosin staining was performed for the histopathological finding. Brain tissues were fixed in 4% phosphate buffered paraformaldehyde solution, embedded with paraffin, and cut into 4 μm coronal section. The paraffin section were deparaffined in xylene and rehydrated in gradient ethanol from 100% to 70%. The sections were generally stained with hematoxylin and eosin solution. The stained sections were dehydrated using gradient ethanol, slipped with permount (Sigma), and observed under light microscope.

A proteomic analysis was performed as previously described [11]. Right cerebral cortices were homogenized in lysis buffer (8 M urea, 4% CHAPS, ampholytes, and 40 mM Tris–HCl). Protein extracts were centrifuged at 16,000 × $g$ for 20 min at 4 °C. Bradford assay kit (Bio-Rad, Hercules, CA, USA) was used to determine total protein concentration. Total protein (100 μg) was subjected to isoelectric focusing (IEF) on immobilized pH gradient (IPG) gel strips (pH 4–7 and pH 6–9, 17 cm, Bio-Rad). IEF was performed using a multistep protocol: 250 V (15 min), 10,000 V (3 h), and then 10,000 V to 50,000 V using a Protean IEF Cell (Bio-Rad). After equilibration of the IEF strips, strips were applied to gradient gels (7.5–17.5%) for second dimension electrophoresis. Gels were loaded on Protein-II XI electrophoresis equipment (Bio-Rad) at 5 mA for 2 h followed by 10 mA for 10 h at 10 °C. Gels were fixed in 12% acetic acid and 50% methanol for 2 h, and stained with silver solution (0.2% silver nitrate, 0.75 ml/l formaldehyde) for 20 min. Gel images were recorded immediately using Agfar ARCUS 1200™ (Agfar-Gevaert, Mortsel, BEL). Spot analysis was performed using PDQuest 2-D analysis software (Bio-Rad). Gel pieces containing the desired protein spots were excised and destained for MALDI-TOF. Gel particles were digested in trypsin-containing buffer. Extract peptides were analyzed using a Voyager-DE™ STR biospectrometry workstation (Applied Biosystem, Foster city, CA, USA) for MALDI-TOF mass spectrometry. Proteins were identified using the search programs MS-Fit and ProFound. SWISS-PROT and NCBI were used as the target protein sequence databases. The intensity of protein spots was measured using PDQuest software. The spot intensity is described as a ratio of the intensity of that spot in the experimental group relative to that of the corresponding spot in the vehicle + sham group.

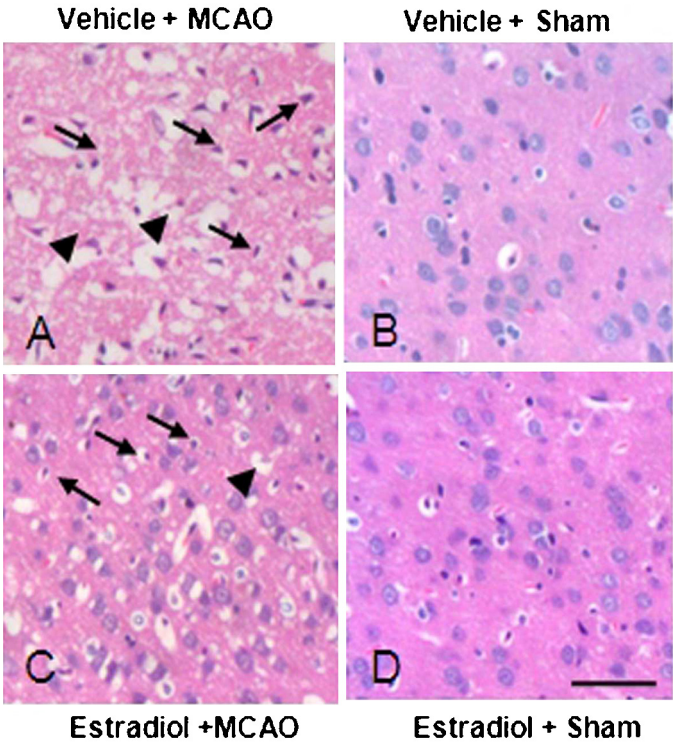


**Fig. 1.** Representative photos of H–E stain in the cerebral cortices from vehicle + middle cerebral artery occlusion (MCAO) (A), vehicle + sham (B), estradiol + MCAO (C), estradiol + sham (D) animals. Arrows indicate apoptotic bodies and arrowhead indicate necrotic changes with scalloped shrunken form in ischemic lesion. Scale bar = 100 μm.

Right cerebral cortices were collected and frozen quickly. Total RNA was extracted with Trizol reagent (Invitrogen, Carlsbad, CA, USA). First strand cDNA synthesis was performed with total RNA (1 μg) and Superscript III reverse transcriptase (Invitrogen) according to the manufacturer's instructions. The following primers were used: hippocalcin primer (forward primer, 5′-ACGCCAACTTCTTCCCCTATG-3′; reverse primer, 5′-AGCCATCAGCGTCTTTGTTT-3′) and actin primer (forward primer, 5′-GGGTCAGAAGGACTCCTACG-3′; reverse primer, 5′-TTTCACTGCGGCTGATGTAG-3′). The amplification program consists of a denaturating at 94 °C for 30 s, annealing at 54 °C for 30 s, and extension at 72 °C for 1 min. Samples were amplified for 30 cycles. PCR products were run on a 1% agarose gel and visualized under UV light.

For Western blot analysis, equal amounts of protein (30 μg) were separated on 10% SDS-polyacrylamide gels and transferred to polyvinylidene fluoride membranes (Millipore, Billerica, MA, USA). Membranes were incubated with 5% skim milk solution for 1 h and then washed in Tris-buffered saline containing 0.1% Tween-20 (TBST). Membranes were probed with the following antibodies: anti-hippocalcin (diluted 1:1000, Abcam, Cambridge, UK) and anti-actin (diluted 1:1000, Santa Cruz Biotechnology, Santa Cruz, CA, USA) as primary antibodies at 4 °C for 15 h. Membranes were then rinsed with TBST and incubated with horseradish peroxidase-conjugated anti-rabbit IgG (1:5000, Pierce, Rockford, IL, USA). Immunoreactivity was detected using enhanced chemiluminescence (ECL Western blotting detection kit, Amersham Pharmacia Biotech, Piscataway, NJ, USA).

Mouse hippocampal cells (HT22) were maintained in Dulbecco's modified Eagle's medium (DMEM, Hyclone Laboratory, Logan, UT, USA) containing 10% heat-inactivated fetal bovine serum