Repression of adenosine triphosphate–binding cassette transporter ABCG2 by estrogen increases intracellular glutathione in brain endothelial cells following ischemic reperfusion injury

Jin A. Shin, Sae Im Jeong, Hye Won Kim, Gyeonghui Jang, Dong-Ryeol Ryu, Young-Ho Ahn, Ji Ha Choi, Yoon-Hee Choi, Eun-Mi Park

1. Introduction

The adenosine triphosphate–binding cassette efflux transporter ABCG2, which is located in the blood-brain barrier limits the entry of endogenous compounds and xenobiotics into the brain, and its expression and activity are regulated by estrogen. This study was aimed to define the role of ABCG2 in estrogen-mediated neuroprotection against ischemic injury. ABCG2 protein levels before and after ischemic stroke were increased in the brain of female mice by ovariectomy, which were reversed by estrogen replacement. In brain endothelial cell line bEnd.3, estrogen reduced the basal ABCG2 protein level and efflux activity and protected cells from ischemic injury without inducing ABCG2 expression. When bEnd.3 cells were transfected with ABCG2 small interfering RNA, ischemia-induced cell death was reduced, and the intracellular concentration of glutathione, an antioxidant that is transported by ABCG2, was increased. In addition, after ischemic stroke in ovariectomized mice, estrogen prevented the reduction of intracellular glutathione level in brain microvessels. These data suggested that the suppression of ABCG2 by estrogen is involved in neuroprotection against ischemic injury by increasing intracellular glutathione, and that the modulation of ABCG2 activity offers a therapeutic target for brain diseases in estrogen-deficient aged women.
brain of patients with Alzheimer's disease with cerebral amyloid angiopathy (Xiong et al., 2009) but was markedly reduced in the cerebral capillaries of patients with capillary cerebral amyloid angiopathy (Carrano et al., 2014), suggesting a role of ABCG2 in amyloid β transport in the brain. A study reported that ABCG2 expression was increased in the peri-infarct area at 3–14 days after stroke in rats and suggested ABCG2 as a marker for the regenerative process (Dazert et al., 2006). Brain endothelial cells are the main component of the BBB and their disruption by ischemic injury results in increased BBB permeability (Jickling et al., 2014). However, the effects of ischemic injury on the activity and expression of ABCG2 in the brain and the role of ABCG2 in brain endothelial cells after ischemic injury are yet to be determined.

Estrogen reduces ABCG2 activity and/or its expression in brain capillaries under normal conditions (Hartz et al., 2010a,b). It has been also demonstrated that estrogen is a potent neuroprotectant against ischemic stroke in rodents (Gibson et al., 2006). Considering that estrogen reduces BBB breakdown and brain endothelial cell death after ischemic insult (Liu et al., 2005; Shin et al., 2016), it is plausible that estrogen-regulated ABCG2 activity and expression might be involved in the neuroprotection exerted by estrogen against ischemic injury.

To determine the role of ABCG2 in brain endothelial cells under ischemic injury, we assessed ABCG2 protein levels in the brain of gonadally intact and ovariectomized female mice after ischemic stroke. The cell viability and level of glutathione, an ABCG2 substrate, were also determined in immortalized mouse brain endothelial cells (bEnd.3) transfected with ABCG2 small interfering RNA (siRNA) following in vitro ischemic injury. In addition, we examined glutathione levels in brain microvessels after ischemic stroke in young and aged female mice.

2. Material and methods

2.1. Animals

Wild-type female C57BL/6 mice purchased from Orient Bio Inc (Seongnam, Republic of Korea) were used. Experiments were performed using young adult (10–11 weeks old) and grossly healthy aged (20 months old) mice. For the measurements of ABCG2 protein levels in the brain (n = 6 per group of young and aged mice without ischemic injury and n = 6 per group of young mice at each time point after ischemic injury) and in brain microvessels (n = 4 in young and aged mice), 58 young and 22 aged mice were used. For immunofluorescence staining of ABCG2 in the brain, 6 young mice (n = 3 per treatment) were used. Eighteen young (n = 6 per group) and 15 aged mice (n = 5 per group) were exposed to ischemic brain injury to measure intracellular glutathione level in the ischemic brain microvessels, and all young and 13 aged mice were survived at 24 hours after ischemic insult. In total, 82 young and 35 aged mice were included in the data analysis. All procedures were approved by the Institutional Animal Care and Use Committee at the Medical School of Ewha Womans University and conformed to international guidelines for the ethical use of experimental animals. Mice were acclimatized to the animal colony, which was kept under a 12-hour light/dark cycle at 22 ± 2 °C for 2 weeks before the experiments. The number of animals used was minimized to reduce animal suffering.

2.2. Ovariectomy and drug administration

Two weeks before inducing middle cerebral artery occlusion (MCAO), female mice were subjected to aseptic bilateral surgical ovariectomy (OVX) via a dorsal incision while under isoflurane anesthesia (1.4%–1.6%). OVX mice were assigned randomly to vehicle and estrogen replacement groups. Subsequently, vehicle (0.1 mL of sesame oil) or E2 (17β-estradiol 3-benzoate, 1 μg in 0.1 mL of sesame oil; Sigma-Aldrich, St. Louis, MO, USA) was administered subcutaneously for 6 days starting 3 days before MCAO. The E2 dose was based from previous studies that demonstrated E2 protective effects in the brains of female mice (Park et al., 2006; Shin et al., 2011).

2.3. Transient MCAO

Transient MCAO was performed by 1 person blinded to the treatment, and the procedures were previously described (Shin et al., 2011). Briefly, mice were anesthetized with isoflurane, and a fiber optic probe was attached to the right parietal bone (2-mm posterior and 5-mm lateral to bregma) and connected to a laser Doppler flowmeter (PeriFlux System 5010, Perimed, Sweden). Cerebral blood flow (CBF) was continuously recorded during MCAO and reperfusion periods with a computer-based data acquisition system (Perisoft, Perimed, Sweden). A 6-0 silicon-coated black monofilament surgical suture (Docol Corporation, Redlands, CA, USA) was inserted into the exposed right external carotid artery, advanced into the internal carotid artery, and wedged into the circle of Willis to obstruct the origin of the middle cerebral artery. The filament was left in place for 30 minutes and then withdrawn to re-establish CBF. Only animals that exhibited a reduction greater than 85% in CBF during MCAO and that recovered by more than 80% after 10 minutes of reperfusion were included in the study. Rectal temperature was maintained at 37.0 ± 0.5 °C with a thermostatically controlled heating pad during surgery and recovery until mice regained consciousness.

2.4. Brain microvessel isolation

The microvessel isolation was based on a protocol described previously (Wu et al., 2003). Briefly, the cerebral hemispheres were collected and rinsed with Dulbecco's Modified Eagle Medium containing 4.5 g/L glucose (DMEM; WELGENE, Daegu, Republic of Korea), supplemented with 2% fetal bovine serum (FBS; ATCC), 100 U/mL penicillin (Gibco, Grand Island, NY, USA), and 100 μg/mL streptomycin (Gibco). Hemispheres were subsequently cut into small pieces and homogenized with Dounce tissue grinder (Fisher Scientific, Pittsburgh, PA, USA), suspended in 15% dextran (average molecular weight 70,000; Sigma, St. Louis, MO, USA), and then centrifuged for 15 minutes at 12,000 rpm at 4 °C. The pellets were digested in 10 mL of 0.1% collagenase/dispase (Roche, Mannheim, Germany) supplemented with 2% FBS for 4 hours at 37 °C with occasional agitation. After samples were centrifuged for 15 minutes at 4000 rpm, the pellets were resuspended in 45% Percoll (GE Healthcare, Upssala, Sweden) in phosphate-buffered saline (PBS) and centrifuged for 15 minutes at 12,000 rpm at 4 °C. The microvessel layers located in the top layer were collected and washed once with PBS and were subsequently used for Western blot analysis.

2.5. Cell culture and treatments

The bEnd.3 cells (ATCC, Manassas, VA, USA) were cultured on 60-mm dishes (for Western blot and glutathione assay), 24-well plates (for cytotoxicity assay and immunofluorescence staining), and 96-well plates (for cell viability assay) at a seeding density of 5.0 × 10^4 to 5.0 × 10^5 cells/well. Cells were maintained in a humidified incubator (95% air/5% CO2; Thermo Scientific, Rockford, IL, USA) with DMEM containing 10% FBS, 100 U/mL penicillin, and 100 μg/mL streptomycin (normal DMEM) at 37 °C. Cells were pretreated with 1 μM E2 dissolved in dimethyl sulfoxide (DMSO; Sigma-Aldrich) 48 hours before and during oxygen-glucose deprivation (OGD) and for 18 hours after OGD (Fig. 5). The dose of E2 was within...
Fig. 5. Effects of ABCG2 knockdown on bEnd.3 cells exposed to oxygen-glucose deprivation (OGD). (A) Experimental timeline of siRNA transfection, vehicle (0.1% DMSO), or 17β-estradiol 3-benzoate (E2, 1 nM) treatment and 6 hours of oxygen-glucose deprivation (OGD) with 18-hour reperfusion in bEnd.3 cells. (B) Western blots of ABCG2 protein and its quantification in bEnd.3 cells transfected with mock, control siRNA (Cont), or ABCG2 siRNA (n = 6 per transfection). * \( p < 0.05 \) versus Mock and Cont. (C) Representative histogram of intracellular accumulation of pheophorbide a (PhA) and mean fluorescence intensity of PhA (arbitrary unit) in cells transfected with mock, control siRNA (Cont), or ABCG2 siRNA. Five sets of experiments were performed independently. * \( p < 0.05 \) versus Mock and Cont. MTT assay (D) and LDH assay (E) of bEnd.3 cells transfected with mock, control siRNA (Cont), or ABCG2 siRNA under normal culture condition (control) or exposed to 18 hours after 6-hour OGD (n = 6 per transfection). * \( p < 0.05 \) versus Mock and control siRNA under OGD. (F) Western blots of ABCG2 protein and its quantification in bEnd.3 cells transfected with mock, control siRNA (Cont), or ABCG2 siRNA at 18 hours after 6-hour OGD (n = 6 per transfection). * \( p < 0.05 \) versus Mock and Cont. MTT assay (G) and LDH assay (H) of vehicle (0.1% DMSO) or E2 (1 nM)-treated bEnd.3 cells after transfection with mock, control siRNA (Cont), or ABCG2 siRNA at 18 hours after 6-hour OGD (n = 6 per transfection). * \( p < 0.05 \) versus mock and control siRNA with vehicle treatment; # \( p < 0.05 \) versus ABCG2 siRNA with vehicle treatment. Abbreviations: DMSO, dimethyl sulfoxide; LDH, lactate dehydrogenase; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; siRNA, small interfering RNA.
physiological concentrations and was based on previous reports (Cornil et al., 2006; Shin et al., 2016). The final concentration of DMSO was 0.1% (v/v), and DMSO alone was used for vehicle treatment.

2.6. Oxygen-glucose deprivation

OGD was induced as previously described (Shin et al., 2016). Confluent cells were washed with fresh media twice, replaced by a glucose-free Earle’s balanced salt solution (pH 7.4), containing NaCl [116 mM], KCl [5.4 mM], MgSO4 [0.8 mM], 1.0 NaH2PO4 [1.0 mM], CaCl2 [1.8 mM] and NaHCO3 [26 mM] pre-gassed with 95% N2/5% CO2, and incubated in a humidified anaerobic incubator (Multi Gas Incubator; Astec, Fukuoka, Japan) under 94% N2/5% CO2/1% O2 for 6 hours. Subsequently, culture medium was replaced with fresh normal DMEM and maintained in an incubator under 95% air/5% CO2 until the defined experimental time points. Control cells were incubated with Earle’s solution supplemented with 4.5 g/L glucose for 6 hours, and were subsequently maintained in a manner identical to that of OGD (with normal DMEM under 95% air/5% CO2).

2.7. Cell viability and cytotoxicity measurements

Cell viability was determined by the reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) to a blue formazan crystal by adding 10 μL of MTT (5 mg/mL; Sigma-Aldrich) to cells at 6 hours of OGD or 18 hours after 6 hours OGD. After 4 hours incubation at 37 °C, culture medium was removed, DMSO added, and the plate was agitated to solubilize formazan crystals on a shaker. Optical density (OD) of the plates was measured as the absorbance at a wavelength of 540 nm by a microplate reader (Versa Max; Molecular Devices, CA, USA), and cell viability was expressed as the percentage of OD for cells exposed to OGD over the OD of control cells. To measure cell death and cell lysis, the lactate dehydrogenase (LDH) activity in the cell culture media was measured using a commercially available kit (Cytotoxicity Detection KitPLUS; Roche, Mannheim, Germany). The absorbance intensity of LDH activity was measured at 490 nm by a microplate reader (Versa Max; Molecular Devices, CA, USA), and cell viability was expressed as the percentage of OD for cells exposed to OGD over the OD of control cells. To measure cell death and cell lysis, the lactate dehydrogenase (LDH) activity in the cell culture media was measured using a commercially available kit (Cytotoxicity Detection KitPLUS; Roche, Mannheim, Germany). The absorbance intensity of LDH activity was measured at 490 nm by a microplate reader, and cytotoxicity (%) was calculated as (experimental value – low control)/ (high control – low control) × 100, with low control being the LDH activity of untreated cells and high control being the maximum LDH activity of cells treated with 1% Triton X-100.

2.8. Small interfering RNA transfection

Cells at 50%–60% confluence were transfected with control or ABCG2 siRNA (200 nM, each; Santa Cruz Biotechnology, Santa Cruz, CA, USA) using a transfection reagent (HiPerfect; Qiagen, Germantown, MD, USA) according to the manufacturer’s instructions. The concentration of ABCG2 siRNA was based on a previous study (Jin et al., 2014). Three hours after transfection, cells were maintained in a fresh medium for 24 hours before further experiments.

2.9. Flow cytometric efflux assay

Pheophorbide a (PhA; Cayman Chemical, Ann Arbor, MI, USA) is an ABCG2-specific substrate and was used as a probe for ABCG2 function (Röbben et al., 2004). Vehicle or E2-treated cells or cells transiently transfected with control or ABCG2 siRNA were trypsinized, detached, and centrifuged at 4000 rpm for 5 minutes. Cells resuspended in culture medium containing 1 μM PhA were incubated for 30 minutes in an incubator. Subsequently, cells were washed twice with cold PBS, collected, and were kept on ice with light protection until flow cytometry analysis. Intracellular accumulation of PhA was analyzed with the BD FACScnt flow cytometry system (BD biosciences, Mountain View, San Jose, CA, USA) by using 488-nm argon laser and 530-nm bandpass filter. Cells were gated using single cell characteristics and at least 10,000 gated events were collected. Mean fluorescence intensity values for each histogram were measured using CellQuest software (BD biosciences).

2.10. Western blot analysis

To examine ABCG2 protein levels in the brain, naïve control mice, mice at 6 hours, and mice at 24 hours reperfusion after MCAO were euthanized. The right cortex and isolated brain microvessels of control mice, ipsilateral cortex of MCAO mice, and bEnd.3 cells were used in the Western blot assay. Tissues and cells were lysed in sodium dodecyl sulfate buffer (50 mM Tris–HCl, 1% Igepal CA-630, 0.25% deoxycholic acid, 150-mM NaCl, 1-mM ethylene-diaminetetra acetic acid, 1-mM sodium dodecyl sulfate; pH 7.4) containing a protease inhibitor cocktail (Complete Mini, Roche, Mannheim, Germany), and proteins were isolated and measured using the Bradford assay (Bio-Rad Laboratories, Hercules, CA, USA). A total of 80 μg of protein from the cortex, 15 μg from brain microvessels, and 30 μg from cells were loaded, electrophoresed, and transferred to Trans-Blot Turbo Transfer membranes (Bio-Rad Laboratories) as previously described (Shin et al., 2015). Membranes were blocked for 1 hour in tris-buffered saline containing 0.1% Tween-20% and 10% dry milk. Subsequently, membranes were incubated overnight with antibodies against ABCG2 (1:500; Santa Cruz Biotechnology, Santa Cruz, CA, USA) or actin (1:1000; Santa Cruz Biotechnology). After 1-hour incubation with hors eradish peroxidase-conjugated secondary antibodies, protein bands were visualized with the Western Blotting Luminol Reagent (Santa Cruz Biotechnology). For quantification, the density of each band was normalized to the density of actin using Image J (version 1.37, NIH, Bethesda, MD, USA).

2.11. Double immunofluorescence staining

Brains perfused with normal saline followed by cold 4% formaldehyde in 0.1 M sodium phosphate buffer (pH 7.2) were incubated overnight in fixative and stored in a 30% sucrose solution. Using a cryostat, serial coronal brain sections (20-μm thick, 600-μm intervals) were cut throughout the region spanning +1.4 to −1.0 mm from the bregma and mounted on gelatin-coated slides. Cells grown in 24-well plates were fixed with cold methanol and acetone (1:1 ratio) for 20 minutes at −20 °C. Brain sections and cells were incubated in tris-buffered saline containing 0.1% Triton X-100, 5% normal serum, and 1% bovine serum albumin for 1 hour and then incubated with primary antibodies against ABCG2 (1:500; Santa Cruz Biotechnology) and against CD31 (1:100; BD Biosciences) overnight at 4 °C. On the following day, samples were incubated with secondary antibody conjugated to fluorescein isothiocyanate (1:1000; Vector Laboratories, Inc, Burlingame, CA, USA) and Alexa Fluor 555 (1:1000; Molecular Probes, Eugene, OR, USA) for 1 hour at room temperature. After washing, sections and cells were mounted with VECTASHIELD mounting medium (Vector Laboratories, Inc). Fluorescence images were obtained using a confocal microscope (LSM5 PASCAL; Carl Zeiss).

2.12. Glutathione assay

Extracellular and intracellular glutathione were measured using the QuantiChrom Glutathione Assay Kit (BioAssay Systems, Hayward, CA, USA) according to the manufacturer’s procedures. Conditioned medium and lysates from bEnd.3 cells were collected for measurements of extracellular and intracellular glutathione, respectively. In addition, to measure intracellular glutathione in brain microvessels after ischemic stroke, lysates of brain...
microvessels from ipsilateral hemispheres were collected. Protein titration was performed to normalize the glutathione level. The absorbance at 412 nm of the reaction of 5,5'-dithiobis(2-nitrobenzoic acid) with glutathione was measured to determine the glutathione concentration.

2.13. Statistical analysis

Data are expressed as mean ± standard error of the mean. Comparisons between 2 groups were analyzed using unpaired Student's t-tests, and comparisons of more than 2 groups were analyzed using one-way analysis of variance, followed by Tukey's post hoc test (Prism 6, GraphPad Software, Inc, La Jolla, CA, USA). Statistical significance was set at \( p < 0.05 \).

3. Results

3.1. Estrogen reduces the level of ABCG2 protein in the brain of female mice

To test the effect of estrogen on ABCG2 expression in the brain, the protein level of ABCG2 was examined in the brain of young and aged female mice with 1 of 3 different conditions: gonadally intact, OVX treated with vehicle, or OVX treated with E2. Compared with gonadally intact mice, the ABCG2 protein level in both young and aged mice was significantly increased by OVX \( (p < 0.01) \), which was repressed by E2 treatment (Fig. 1A and B). Previous studies have demonstrated that the plasma level of E2 is significantly lower in aged female mice than in young mice, and that ABCG2 are preferentially localized in capillary endothelial cells in the mouse brain (Jeong et al., 2016; Tachikawa et al., 2005). Based on these previous findings, the protein level of ABCG2 was examined in microvessels isolated from the brains of young and aged mice. The results indicated a significant increase of ABCG2 expression in microvessels of estrogen-deficient aged female mice \( (p < 0.01\text{ vs. young; Fig. 1C}) \). The suppressive effect of E2 on the ABCG2 protein level was further examined by double immunofluorescence staining in the brains of young OVX mice treated either with vehicle or E2. ABCG2 expression was detected in the luminal side of brain vessels stained with the endothelial cell marker CD31 in mice treated with vehicle but not in mice treated with E2 (Fig. 1D). These results indicate that the ABCG2 protein level is suppressed by estrogen in brain microvessels of female mice.

3.2. Estrogen prevents the increase of ABCG2 protein level in the brain after ischemic stroke

To determine the effect of estrogen on the ischemia-induced ABCG2 protein expression in the brain, ABCG2 protein levels were examined in the brain of young female mice at 6 hours and 24 hours...
after MCAO. In the brain of gonadally intact mice, ABCG2 expression decreased, rather than increasing, at 6 hours and was subsequently restored at 24 hours after MCAO. These differences, however, were not statistically significant compared with naïve controls (Fig. 2A). In contrast, the level of ABCG2 significantly increased in the brain of OVX mice treated with vehicle both at 6 hours \( (p < 0.01) \) and 24 hours \( (p < 0.05) \) after MCAO compared with controls (Fig. 2B). No increase of ABCG2 expression was observed in the ischemic brain of OVX mice treated with E2 (Fig. 2C). These results indicate that estrogen prevents ischemia-induced ABCG2 expression in young OVX mice.

3.3. Estrogen suppresses basal ABCG2 expression and activity in brain endothelial cells

To determine the effect of estrogen-induced inhibition of ABCG2 in brain endothelial cells, bEnd.3 cells were treated with vehicle or E2 (1 nM) for 72 hours (Fig. 5A). Similarly to our in vivo results, the ABCG2 protein levels decreased in cells treated with E2 \( (p < 0.01) \) vs. both no treatment and vehicle; Fig. 3A), which was also demonstrated by immunofluorescence staining against ABCG2 (Fig. 3B). To determine ABCG2 activity, we measured the intracellular accumulation of PhA, which is a substrate of ABCG2. Compared with vehicle, E2 significantly increased the PhA fluorescence intensity in cells, indicating a decreased efflux activity of ABCG2 (1.72-fold change, \( p < 0.01 \) vs. vehicle; Fig. 3C). These results indicate that estrogen inhibits ABCG2 both at the level of its expression and activity in brain endothelial cells.

3.4. Estrogen mitigates OGD-induced cell death and reduces ABCG2 expression in brain endothelial cells

To determine whether ABCG2 inhibition was associated with estrogen-mediated protective effects, vehicle or E2-treated bEnd.3 cells were exposed to 6-hour OGD and 18 hours after 6-hour OGD. Cell viability measured by the MTT assay was decreased, while cytotoxicity measured by the LDH assay was increased in vehicle-treated cells following OGD compared with control cells. However, E2 treatment significantly reduced the OGD-induced cell death \( (p < 0.01 \text{ vs. no treatment and vehicle for both MTT and LDH assays; Fig. 4A and B}) \). Similarly to our in vivo results following ischemia, ABCG2 protein levels significantly increased at 18 hours after OGD in vehicle-treated cells \( (p < 0.01 \text{ vs. control; Fig. 4C}) \) but not in cells treated with E2 (Fig. 4D). These results suggest that the repression of OGD-induced ABCG2 expression in brain endothelial cells is related to the protective effects of estrogen against OGD.

3.5. Suppression of ABCG2 mitigates OGD-induced brain endothelial cell death

To determine whether ABCG2 inhibition contributes to estrogen-mediated protection, bEnd.3 cells were transiently transfected with ABCG2 siRNA (Fig. 5A). The results indicated a significant decrease in the basal expression of ABCG2 compared with control siRNA (42% reduction, \( p < 0.01 \); Fig. 5B). In addition, intracellular PhA accumulation was significantly increased in cells transfected with ABCG2 siRNA (1.86-fold change, \( p < 0.01 \) vs. control siRNA; Fig. 5C), indicating that ABCG2 siRNA effectively represses ABCG2 in bEnd.3 cells. To determine the effect of downregulated ABCG2 function on cell viability, cells transfected with ABCG2 siRNA were exposed to OGD. Our results indicated that ABCG2 siRNA-transfected cells were resistant to the OGD insult compared with cells transfected with control siRNA as evidenced by the MTT and LDH assays (both \( p < 0.01 \); Fig. 5D and E). At the same time, no increase of ABCG2 expression was observed at 18 hours after OGD in cells transfected with ABCG2 siRNA \( (p < 0.01 \text{ vs. control siRNA; Fig. 5F}) \). These data suggest that the downregulation of ABCG2 function mitigates cell damage associated with OGD. We further examined whether ABCG2 inhibition was one of the protective mechanisms of E2 against OGD. When cells transfected with
ABCG2 siRNA treated with vehicle or E2 were exposed to OGD, the protective effects of ABCG2 inhibition were significantly enhanced by E2 treatment ($p < 0.01$ vs. vehicle-treated cells transfected with ABCG2 siRNA for both MTT and LDH assays; Fig. 5G and H). Enhanced survival observed after E2 treatment in ABCG2 siRNA-transfected cells may have resulted from further downregulation of ABCG2 activity or other protective effects of estrogen, such as its anti-inflammatory and antiapoptotic actions against ischemic insults (Strom et al., 2011). Altogether, these data indicate that ABCG2 inhibition plays a role in estrogen-mediated protective effects against ischemic injury.

### 3.6. Suppression of ABCG2 increases intracellular glutathione in brain endothelial cells

ABCG2 transports glutathione, which is an endogenous antioxidant (Brechbuhl et al., 2010). It has been suggested that the prevention of intracellular glutathione depletion reduces endothelial cell loss following ischemic reperfusion (Ronaldson and Davis, 2015). To determine whether the protective effect mediated by ABCG2 inhibition was associated with glutathione levels, extracellular and intracellular glutathione levels were measured in cells transfected with ABCG2 siRNA. Under normal conditions, no changes in extracellular glutathione levels were observed between transfections (Fig. 6A); in contrast, the level of intracellular glutathione increased in cells transfected with ABCG2 siRNA ($p < 0.05$ vs. control siRNA; Fig. 6B). OGD insult induced the increase of both extracellular and intracellular glutathione levels in bEnd.3 cells treated with mock and transfected with control siRNA (see the scale difference of ordinates in Fig. 6A and C). In cells with ABCG2 siRNA transfection, extracellular glutathione increased to a lesser extent, while intracellular glutathione markedly increased compared with control siRNA transfection ($p < 0.01$ for both extracellular and intracellular glutathione; Fig. 6C and D). We further examined intracellular glutathione levels in the isolated brain microvessels from the ischemic brain of young female mice at 24 hours after MCAO, the time point that protein level of ABCG2 was increased in OVX mice (Fig. 2B). Intracellular glutathione levels were decreased in OVX mice ($62.27 \pm 1.42$ nmol/mg) compared with that of gonadally intact mice ($90.05 \pm 2.55$ nmol/mg, $p < 0.01$; Fig. 6E). E2 treatment reversed this decrease of glutathione levels in OVX mice ($105.7 \pm 4.21$ nmol/mg, $p < 0.01$ vs. vehicle). We also examined levels of intracellular glutathione in the ischemic brain of naturally senescent estrogen-deficient mice. As expected, intracellular glutathione levels were lower in the ischemic brain microvessels of aged mice ($69.47 \pm 2.20$ nmol/mg) compared to that of young mice. The results might be related to the decreased E2 levels in aged female mice (Jeong et al., 2016). The reduction of glutathione levels was further decreased by OVX ($55.98 \pm 0.75$ nmol/mg, $p < 0.01$ vs. intact), but E2 treatment significantly increased glutathione level ($77.72 \pm 2.24$ nmol/mg) compared to those of both gonadally intact ($p = 0.04$) and OVX mice ($p < 0.01$; Fig. 6F). Altogether, these in vitro and in vivo results suggest that the increase of intracellular glutathione resulting from E2 inhibition of ABCG2 is involved in the survival of brain endothelial cells following ischemic reperfusion injury (Fig. 6E).
ischemia-induced kidney dysfunction and tissue damage were observed in estrogen-treated brains and brain endothelial cells that upregulate ABCG2 expression (Krishnamurthy et al., 2004; Shin et al., 2013, 2016). However, such upregulation was not observed in brain endothelial cells in the absence of estrogen. Ischemia-induced hypoxia-inducible factor 1α, which binds and activates the ABCG2 promoter, may be involved in the upregulation of ABCG2 expression (Krishnamurthy et al., 2004; Shin et al., 2013, 2016). However, such upregulation was not observed in estrogen-treated brains and brain endothelial cells that were resistant to ischemic insult. In addition, cells transfected with ABCG2 siRNA exhibited increased cell viability after OGD, even without estrogen treatment. Our results indicated that inhibition, rather than upregulation, of ABCG2 is beneficial for cell survival following ischemic insult. Similarly to our findings, kidneys of ABCG2-deficient mice were protected against ischemic injury, and ischemia-induced kidney dysfunction and tissue damage were reduced in wild-type mice grafted with ABCG2-deficient bone marrow (Huls et al., 2010). It was suggested that the increased infiltration of bone marrow cells, such as monocytes, granulocytes, and endothelial progenitor cells, into the renal tissue was behind the protective effect of ABCG2 inhibition (Huls et al., 2010). As the decrease in ABCG2 efflux function results in the accumulation of its substrates, we focused on the level of an endogenous substrate of ABCG2, antioxidant glutathione, in the brain endothelial cells. As shown in cells transfected with ABCG2 siRNA, the increase of intracellular glutathione level might help to cells to overcome effectively oxidative stress and thereby promote cell survival following ischemic reperfusion injury. Our results were in agreement with a recent study showing that the vulnerability of ABCG2 overexpressing kidney epithelial cells to oxidants might be due to impaired antioxidant defense because the content of glutathione was decreased in these cells compared with control cells (Krzyzanowski et al., 2014). Altogether, we suggested that the upregulated ABCG2 expression and activity are involved in brain endothelial cell damage and BBB breakdown after ischemic stroke. However, because ABCG2 was suggested as a marker for the regenerative process in the ischemic brain (Dazert et al., 2006), the long-term effects of ABCG2 expression changes should be further determined in the brain following ischemic stroke.

4. Discussion

The present study demonstrated that estrogen reduces both the basal level and ischemia-induced upregulation of ABCG2 protein expression both in vivo (brain) and in vitro (brain endothelial cells). Reduction of ABCG2 activity was determined in brain endothelial cells by the efflux assay. Cells transfected with ABCG2 siRNA were resistant to OGD, and the protective effect of ABCG2 inhibition was further increased by E2 treatment. As the intracellular glutathione level increased in cells transfected with ABCG2 siRNA, our findings suggested that ABCG2 suppression exerts protective effects in cells exposed to ischemic reperfusion injury via increasing the levels of glutathione.

In peripheral cells, hypoxic or ischemic stimuli upregulates the expression of ABCG2. This increase is regarded as a cytoprotective mechanism because ABCG2 inhibition by gene deletion or pharmacological inhibitors has been reported to decrease cell viability (Higashikuni et al., 2010; Krishnamurthy et al., 2004; Liu et al., 2013; van Dijk et al., 2012). We also observed an increase of ABCG2 in the ischemic brain and endothelial cells in the absence of estrogen. Ischemia-induced hypoxia-inducible factor 1α, which binds and activates the ABCG2 promoter, may be involved in the upregulation of ABCG2 expression (Krishnamurthy et al., 2004; Shin et al., 2013, 2016). However, such upregulation was not observed in estrogen-treated brains and brain endothelial cells that were resistant to ischemic insult. In addition, cells transfected with ABCG2 siRNA exhibited increased cell viability after OGD, even without estrogen treatment. Our results indicated that inhibition, rather than upregulation, of ABCG2 is beneficial for cell survival following ischemic insult. Similarly to our findings, kidneys of ABCG2-deficient mice were protected against ischemic injury, and ischemia-induced kidney dysfunction and tissue damage were reduced in wild-type mice grafted with ABCG2-deficient bone marrow (Huls et al., 2010). It was suggested that the increased infiltration of bone marrow cells, such as monocytes, granulocytes, and endothelial progenitor cells, into the renal tissue was behind the protective effect of ABCG2 inhibition (Huls et al., 2010). As the decrease in ABCG2 efflux function results in the accumulation of its substrates, we focused on the level of an endogenous substrate of ABCG2, antioxidant glutathione, in the brain endothelial cells. As shown in cells transfected with ABCG2 siRNA, the increase of intracellular glutathione level might help to cells to overcome effectively oxidative stress and thereby promote cell survival following ischemic reperfusion injury. Our results were in agreement with a recent study showing that the vulnerability of ABCG2 overexpressing kidney epithelial cells to oxidants might be due to impaired antioxidant defense because the content of glutathione was decreased in these cells compared with control cells (Krzyzanowski et al., 2014). Altogether, we suggested that the upregulated ABCG2 expression and activity are involved in brain endothelial cell damage and BBB breakdown after ischemic stroke. However, because ABCG2 was suggested as a marker for the regenerative process in the ischemic brain (Dazert et al., 2006), the long-term effects of ABCG2 expression changes should be further determined in the brain following ischemic stroke.

Estrogen reduces oxidative stress in the brain via direct or indirect mechanisms (Strom et al., 2011). Estrogen directly scavenges reactive oxygen species at supraphysiological concentration. In contrast, estrogen in physiological concentration, which was used in the present study, indirectly reduces oxidative stress by upregulating antioxidative defense mechanisms such as glutathione content and superoxide dismutase activity in normal and ischemic brains of ovarietomized rats (Feng and Zhang, 2005; Ozacmak and Sayan, 2009; Strom et al., 2011; Topcuoglu et al., 2009). In the aspect
Fig. 6. Effect of ABCG2 inhibition on glutathione levels in bEnd.3 cells. Extracellular (A) and intracellular glutathione levels (B) in cells transfected with mock, control siRNA (Cont), and ABCG2 siRNA under normal control condition (n = 6 per transfection). *p < 0.05 versus Mock and Cont. Extracellular (C) and intracellular glutathione levels (D) in cells transfected with mock, control siRNA (Cont), and ABCG2 siRNA at 18 hours after 6 hours of oxygen-glucose deprivation (OGD; n = 6 per transfection). *p < 0.05 versus Mock and Cont. Intracellular glutathione levels in brain microvessels at 24 hours after middle cerebral artery occlusion (MCAO) of gonadally intact, bilateral surgical ovariectomy (OVX) with vehicle (OVX + Veh) or 17β-estradiol 3-benzoate (E2) (OVX + E2) treated young (n = 6 per group) (E) and aged (n = 3–5 per group) (F) female mice. Vehicle (sesame oil) or E2 (1 mg per day) was treated for 6 days starting 3 days before MCAO. *p < 0.05 versus intact; #p < 0.05 versus OVX + Veh. (G) A scheme for a hypothesis of the effect of ABCG2 suppression by estrogen in brain endothelial cells. Ischemic injury induces ABCG2 expression and activity, which transports antioxidant glutathione extracellularly. Depletion of intracellular glutathione makes cells susceptible to ischemia-induced oxidative stress. In the presence of 17β-estradiol 3-benzoate (E2), not only the basal levels but also ischemia-induced expression and activity of ABCG2 are suppressed, thereby resulting in the increase of intracellular glutathione concentration. ABCG2 inhibition may be a part of protective and antioxidative effects of E2 against ischemic stroke. Abbreviation: siRNA, small interfering RNA.
of controlling glutathione levels in cells, estrogen has been reported to increase the basal concentration of glutathione in cultured neuron and endothelial cells (Dimitrova et al., 2002; Schmidt et al., 2002). In human studies, there is evidence of changes in glutathione levels by estrogen. Glutathione level in whole blood from premenopausal women, who were in a state of surgical menopause by oophorectomy, decreased remarkably compared to baseline premenopausal women, who were in a state of surgical menopause during the first day of menstruation, when estrogen levels were considered to be higher, was higher than that measured at other days in menstruation (Sheng-Huang et al., 2015). Consistently, our in vivo experiments also showed different intracellular glutathione levels in brain microvessels after ischemic stroke between OVX mice with and without estrogen (Fig. 6E and F), supporting that estrogen may affect intracellular glutathione levels in women.

However, how estrogen regulates intracellular glutathione has not been clearly defined. Based on our results, we suggest that the suppression of ABCG2 activity by estrogen is associated with the increase in intracellular glutathione concentration in females. Our findings are the first evidence showing a link between ABCG2 inhibition and estrogen-mediated antioxidative and protective effects in brain endothelial cells following ischemic injury.

As a mechanism of estrogen-mediated ABCG2 inhibition, pro-teosomal degradation of ABCG2 via the estrogen receptors has been suggested in brain endothelial cells (Hartz et al., 2010a). The use of estrogen to increase drug transport into the brain has also been proposed. However, the physiological role of ABCG2 suppression is still not well defined in women. Given that ABCG2 transports a broad range of substrates including xenobiotics (Iorio et al., 2016), the question remains as to how to prevent the entry of other ABCG2 substrates into the brain of women before menopause. The answer can be obtained from mice lacking ABCG2, which are viable and have a normal lifespan (Jonker et al., 2000). It is suggested that another ABC transporter p-glycoprotein (ABCB1) shares the substrates of ABCG2 and that p-glycoprotein and ABCG2 may thus work together at the level of BBB (Iorio et al., 2016). It can be assumed that other efflux transporters may also control the penetration of substrates into the brain when the ABCG2 function is inhibited in the brain. Given that several regulatory pathways are involved in the control of ABC transporters and that the regulatory processes are complex in brain endothelial cells (Qosa et al., 2015), further studies are warranted to determine the effects of estrogen on other transporters and their substrates.

In conclusion, ABCG2 expression in the brain of female mice was downregulated by estrogen, leading thus to the increase of intracellular glutathione and the protection of brain endothelial cells from ischemic injury. We suggested that increased intracellular glutathione is a contributing factor for the beneficial effects of estrogen-mediated ABCG2 inhibition. Our findings may contribute to new insights on the beneficial effects of estrogen and the role of ABCG2 in brain diseases. We further suggest that modulation of ABCG2 activity can be a therapeutic target for the management of brain diseases in estrogen-deficient aged women.

Disclosure statement
The authors report no conflicts of interest.

Acknowledgements

This study was supported by the National Research Foundation of Korea (NRF) grants funded by the Korea government (MSIP) (2010-0027945 and 2014R1A2A1A11051461).

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