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Original Article

Glutathione Protects Brain Endothelial Cells from Hydrogen Peroxide-Induced Oxidative Stress by Increasing Nrf2 Expression

Juhyun Song[1], So Mang Kang[1,2], Won Taek Lee[1], Kyung Ah Park[1], Kyoung Min Lee[3] and Jong Eun Lee[1,2]*

[1]Department of Anatomy, Yonsei University College of Medicine, [2]BK21 Plus Project for Medical Sciences, Yonsei University College of Medicine, Seoul 120-572, [3]Department of Neurology, Seoul National University College of Medicine, Seoul 110-744, Korea

Glutathione (GSH) protects cells against oxidative stress by playing an antioxidant role. Protecting brain endothelial cells under oxidative stress is key to treating cerebrovascular diseases and neurodegenerative diseases including Alzheimer's disease and Huntington's disease. In present study, we investigated the protective effect of GSH on brain endothelial cells against hydrogen peroxide (H_2O_2). We showed that GSH attenuates H_2O_2-induced production of nitric oxide (NO), reactive oxygen species (ROS), and 8-Oxo-2'-deoxyguanosine (8-OHdG), an oxidized form of deoxiguanosine. GSH also prevents H_2O_2-induced reduction of tight junction proteins. Finally, GSH increases the level of nuclear factor erythroid 2-related factor 2 (Nrf2) and activates Nrf2-mediated signaling pathways. Thus, GSH is a promising target to protect brain endothelial cells in conditions of brain injury and disease.

Key words: glutathione (GSH), murine brain endothelial cells (bEnd.3 cells), apoptosis, hydrogen peroxide (H_2O_2), Reactive oxygen species (ROS), nuclear factor erythroid 2-related factor 2 (Nrf2)

INTRODUCTION

Glutathione (GSH) is a ubiquitous thiol-containing tripeptide that plays a cellular protective role under oxidative stress [1]. GSH modulates the response of a cell to redox changes by regulating antioxidant gene expression [2, 3]. Oxidative stress contributes to the progression of neurodegenerative diseases [4] and stroke [5]. Several studies demonstrated that GSH prevents the apoptotic death of endothelial cells in response to oxidative stress [6, 7]. The GSH-dependent antioxidant pathway plays a role in cell survival [8, 9], and its dysregulation contributes to the initiation and progression of the neurodegenerative diseases including dementia and Huntington's disease [10-12]. The blood brain barrier (BBB) is a barrier formed by endothelial cells [13], which protects against the entry of pathogens and neurotoxic agents into the brain [14]. Disruption of the BBB, by degradation of tight junction proteins, leads to cell death, brain edema and hemorrhage [15]. Nuclear factor erythroid 2-related factor 2 (Nrf2), a leucine zipper redox-sensitive transcription factor, is a key regulator of antioxidant and detoxification gene expression [16-18]. Under oxidative stress, Nrf2 translocates from the cytoplasm to the nucleus and subsequently activates the transcription of antioxidant genes

whose promoters contain the antioxidant response element (ARE) [19-21]. Evidence indicates that Nrf2 promotes cell survival by preventing an increase in ROS [22, 23] in various conditions of oxidative stress [24, 25]. In present study, we investigated whether GSH ameliorates oxidative stress-induced damages of brain endothelial cells. We show that GSH prevents the decrease of tight junction proteins, protects BBB, and activates the Nrf2 pathway. Therefore, our results suggest that GSH is a promising therapeutic target to protect BBB in central nervous system injury and diseases.

MATERIALS AND METHODS

Cell culture

Murine brain endothelial cells (bEnd.3 cells, Manassas, VA, USA) were purchased from ATCC and cultured in Dulbecco's modified Eagle's medium (DMEM, Hyclone Laboratories, UT, USA), supplemented with 10% (v/v) fetal bovine serum (FBS, Hyclone Laboratories, UT, USA) and 100 units/ml of penicillin/streptomycin (Hyclone Laboratories, UT, USA), at 37°C in a humidified atmosphere in the presence of 5% CO_2. Culture medium was changed every 2 days [26].

Drug treatment

GSH (Sigma Aldrich, MO, USA) was melted with PBS. H_2O_2 (Invitrogen, CA, USA) was diluted with PBS. Cultured bEnd.3 cells were divided into six groups as follows: (1) Control group, cultured in completed media, (2) H_2O_2 (500 μM) group, cultured in completed media with H_2O_2 (500 μM) for 24 h, (3) GSH (1 mM) group, cultured in completed media with GSH (1 mM) for 24 h, (4) GSH (10 mM) group, cultured in completed media with GSH (10 mM) for 24 h, (5) H_2O_2 (500 μM) +GSH (1 mM) group, cultured in completed media with H_2O_2 (500 μM) and GSH (1 mM) for 24 h, (6) H_2O_2 (500 μM) +GSH (10 mM) group, cultured in completed media with H_2O_2 (500 μM) and GSH (10 mM) for 24 hr.

Lactate dehydrogenase (LDH) assay

H_2O_2-induced cytotoxicity was quantified by measuring the amounts of LDH released into the culture medium from H_2O_2-injured cells [27, 28]. LDH release (cytotoxicity %) was calculated by dividing the value at the experimental time point by the maximum value. The maximum LDH release was measured after freezing each culture at -70°C overnight, followed by rapid thawing, which induced nearly complete cell damage.

Measurement of nitrite production

Nitrite production was determined using the Griess reaction

[28]. Duplicate 100 μl aliquots of culture media collected from each culture were added to a 96-well plate and mixed with 100 μl modified Griess reagent (Sigma Aldrich, MO, USA). The plate was incubated in the dark for 15 min at room temperature. The absorbance of the reaction product was measured at 540 nm using a microplate reader.

Determination of intracellular ROS

The level of the intracellular ROS in all groups was measured using a fluorescent probe, 2', 7'-dichlorodihydrofluorescein diacetate (DCF-DA; Invitrogen, CA, USA) as previously described [29]. The 1×10^6 cells/ml were seeded in the plate and were treated with H_2O_2 or/and GSH for 24 h. Then, b END.3 cells were treated with 5 μM DCF-DA for 30 min at 37°C, and after washing with PBS, the fluorescence was measured in a microscope (Nikon TS100-F ECLIPSE) equipped with a CCD camera (Hamamatsu Photonics, Shizuoka, Japan) [30].

Western blot analyses

Equal amounts of protein (50 μg) were extracted from bEND.3 cells. They were electrophoresed on 10%~12% SDS-polyacrylamide gels. Separated proteins were electrotransferred to Immunobilon-NC membranes (Millipore). Membranes were blocked for 1 hour at room temperature with 5% skim milk in Tris-buffered saline and 0.1% Tween-20 (TBST). The primary antibodies used were Nrf2 (1:2000, Millipore, MA, USA), extracellular- regulated protein kinases (ERK) (1:1000, Millipore, MA, USA), p-ERK (1:2000, Millipore, MA, USA) and β-actin (1:1000, Santa Cruz, CA, USA). Blots were incubated with the primary antibodies overnight at 4°C. Membranes were washed three times (5 min each) with TBST. The secondary antibodies were anti-rabbit and anti-mouse (1:3000, New England Biolabs, CA, USA) and were incubated for 1 hour at room temperature. After washing with TBST (0.05% Tween 20) three times, immunoreactive signals were detected using chemiluminescence and an ECL detection system (Amersham Life Science, UK) with the LAS 4000 program.

Immunocytochemistry (ICC)

The expression of 8-Oxo-2'-deoxyguanosine (8-OHdG) and Claudin 5 in bEND.3 cells was confirmed by immunocytochemistry. All the experimental groups was washed 3 times with PBS, fixed with 4% paraformaldehyde for 3 hours, and then washed with PBS. bEND.3 cells were permeabilized with 0.025% Triton X-100 and were blocked for 1 hour at room temperature with dilution buffer (Invitrogen, CA, USA). Primary antibody anti-rabbit 8-OHdG (1:500, Santa Cruz, CA, USA), anti-rabbit

Claudin 5 (1:500, Millipore, MA, USA) prepared in the dilution buffer was added to the samples and incubated for 3 hours at room temperature. Primary antibody was removed and cells were washed 3 times for 3 min each with PBS. Later samples were incubated with FITC-conjugated goat anti rabbit second antibodies (1:200, Jackson Immunoresearch, PA, USA) for 2 hours at room temperature. Cells were washed again 3 times for 3 min each with PBS and stained with 1 μg/ml 4′,6-diamidino-2-phenylindole (DAPI) (1:100, Invitrogen, CA, USA) for 10 minutes at room temperature. The fixed samples were imaged using Zeiss LSM 700 confocal microscope (Carl Zeiss, NY, USA).

Statistical analyses

Statistical comparisons were performed using independent t-tests for two groups. SPSS software was used for all analyses. Data were expressed as the mean±S.E.M of 3 independent experiments. Differences were considered significant at #$p < 0.1$, *$p < 0.05$, and **$p < 0.001$.

RESULTS

GSH suppresses H_2O_2-induced cell death

We first evaluated the cytotoxicity of H_2O_2 to bEND.3 cells using LDH assays. Treating cells with 500 μM H_2O_2 resulted in 50% cytotoxicity, which were partially rescued by co-treatment of GSH (1 mM and 10 mM) (Fig. 1A). GSH alone (1 mM and 10 mM) did not affect the rate of cell death. The protective effect of GSH was dose-dependent (32% cytotoxicity in 10 mM, 39% cytotoxicity in 1mM) confirming the specificity. We also measured nitrite levels using Griess reagent to confirm the production of nitric oxide in bEND.3 cells (Fig. 1B). The increase in nitrites in response to the treatment of 500 μM H_2O_2 was partially prevented by 10 mM GSH (12 μM in control, 19 μM in H_2O_2 only, 14 μM in H_2O_2+GSH 10 mM). GSH treatment alone did not change nitrite levels (Fig. 1B). The protective role of GSH was dose-dependent (Fig. 1A, B). As 10 mM GSH treatment alone promoted more cell survival and decreased more nitrite concentration (Fig. 1A, B), we decided to use 10 mM GSH in all the following experiments. Finally, we visualized 8-OHdG by immunocytochemistry to measure oxidative damages to DNA. 8-OHdG level increased in H_2O_2 (500 μM)-treated cells indicating DNA damages. 8-OHdG-positive cells were decreased by co-treatment of GSH (10 mM) (Fig. 1C). Taken together, these results suggest that GSH attenuates H_2O_2-induced damages in bEND.3 cells.

GSH decreases H_2O_2-induced ROS production

We measured ROS levels using DCF-DA reagent, a fluorescent dye that visualizes ROS. DCF-DA-positive cells were increased by H_2O_2 treatment (500 μM), and these were partially blocked by co-treatment of GSH (10 mM) (Fig. 2). Co- treatment of GSH (10 mM) evidently were decreased DCF-DA-positive cells compared with H_2O_2 (500 μM) treatment group. This result indicates that GSH prevents H_2O_2-induced ROS production.

GSH prevents H_2O_2-induced decrease in tight junction proteins

To check the protective effect of GSH on the integrity of tight junctions during oxidative stress, we measured the level of Claudin 5, a tight junction protein, by immunocytochemisty. H_2O_2 (500 μM) treatment decreased the expression of Claudin 5 (Fig. 3). GSH treatment alone was not change the expression of Claudin 5 compared to normal control group. The expression of Claudin 5 was attenuated by GSH co-treatment. This suggests that GSH protects the degradation of Claudin 5 and may protect deterioration of tight junctions in response to oxidative stress.

GSH promotes the Nrf2-mediated signaling

Nrf2 is a key regulator of anti-oxidative responses. To investigate whether Nrf2 signaling is activated in H_2O_2-induced oxidative stress, we first measured the phosphorylation status of ERK by Western blot analysis because the phosphorylation of ERK means the activation of ERK pathway. These results suggested that the protein level of phosphor-ERK/ERK in the H_2O_2 (500 μM) group attenuated compared to those of in the normal control (NC) group (Fig. 4A). The protein level of phosphor-ERK in the H_2O_2 (500 μM) +GSH (10 mM) group was higher than those of the H_2O_2 (500 μM) group (Fig. 4A). Also, we checked the expression of Nrf2 and found that H_2O_2 treatment decreases Nrf2 levels. GSH co-treatment attenuated this decrease. These results suggest that GSH increases Nrf2 levels and activates its downstream signaling pathway.

DISCUSSIONS

Oxidative stress aggravates neurodegenerative diseases [4] and brain injury [5], and excessive ROS and/or reactive nitrogen species (RNS) levels are strongly associated with such states [31-34]. GSH, the most abundant non-protein thiols, decreases ROS levels and activates cellular oxidative stress responses by several mechanisms [35, 36]. In present study, we investigated the protective effect of GSH on H_2O_2- induced oxidative stress in the brain capillary endothelial cells. Our results that GSH inhibits H_2O_2-induced increases in ROS and nitric oxide suggest that GSH may protect brain capillary endothelial cells from oxidative stress.
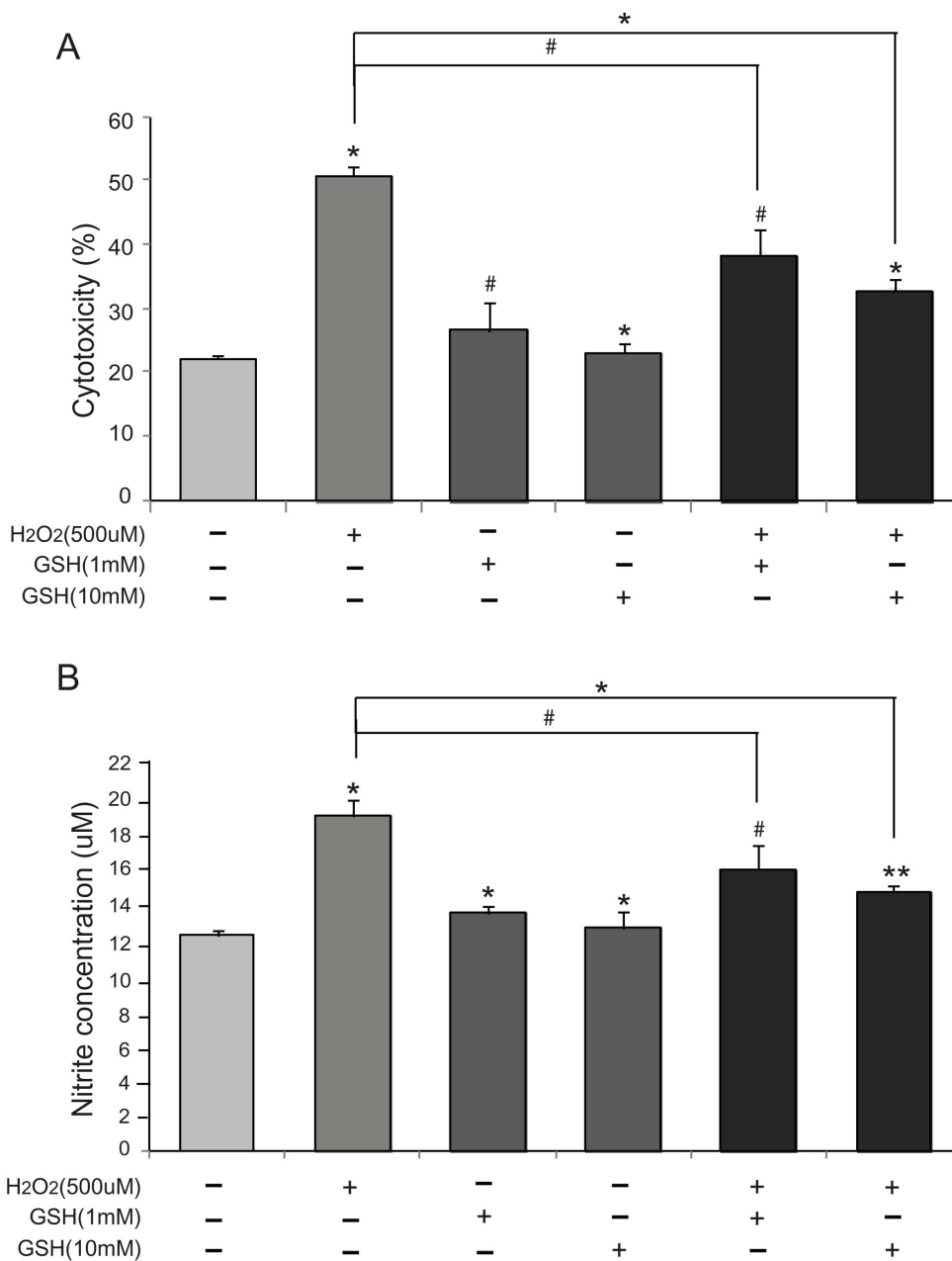
Fig. 1. The effect of GSH H_2O_2-induced cell death. bEDN.3 cells were treated with H_2O_2 (500 μM) and/or GSH (1 mM or 10 mM) for 24 hr. (A) Cytotoxicity was determined by the release of LDH into the culture media. GSH reduced H_2O_2's cytotoxicity. (B) H_2O_2-induced nitrite production was measured by using Griess reagent. GSH reduced H_2O_2-induced nitrite production. Data are expressed as mean±S.E.M. ($^{\#}p < 0.1$, $^{*}p < 0.05$, $^{**}p < 0.001$). (C) 8-OHdG levels were measured by immunocytochemistry. 8-OHdG-positive cells (green color) were increased in the H_2O_2 (500 μM) treatment group compared to the normal control (NC) group. GSH decreased 8-OHdG levels in bEND.3 cells under H_2O_2-induced oxidative stress. Scale bar: 600 μm, 8-Oxo-2'-deoxyguanosine (8-OHdG): green, 4',6-diamidino-2-phenylindole (DAPI): blue.

Several studies demonstrated that GSH inhibits the cell death in oxidative stress [37, 38]. In addition, recent studies demonstrated that GSH inhibits DNA damage of cells against oxidative stress [39] and GSH attenuates the generation of ROS under oxidative stress [40]. Mitochondrial DNA (mt DNA) is one of the cellular components most severely affected by oxidative stress [41]. The oxidative modification of deoxyguanosine to 8-OHdG in mtDNA is the major DNA lesion induced by oxidative stress [42, 43] and is considered as an index of DNA damage [42, 44]. Considering that the elevated level of 8-OHdG is related with increased mtDNA
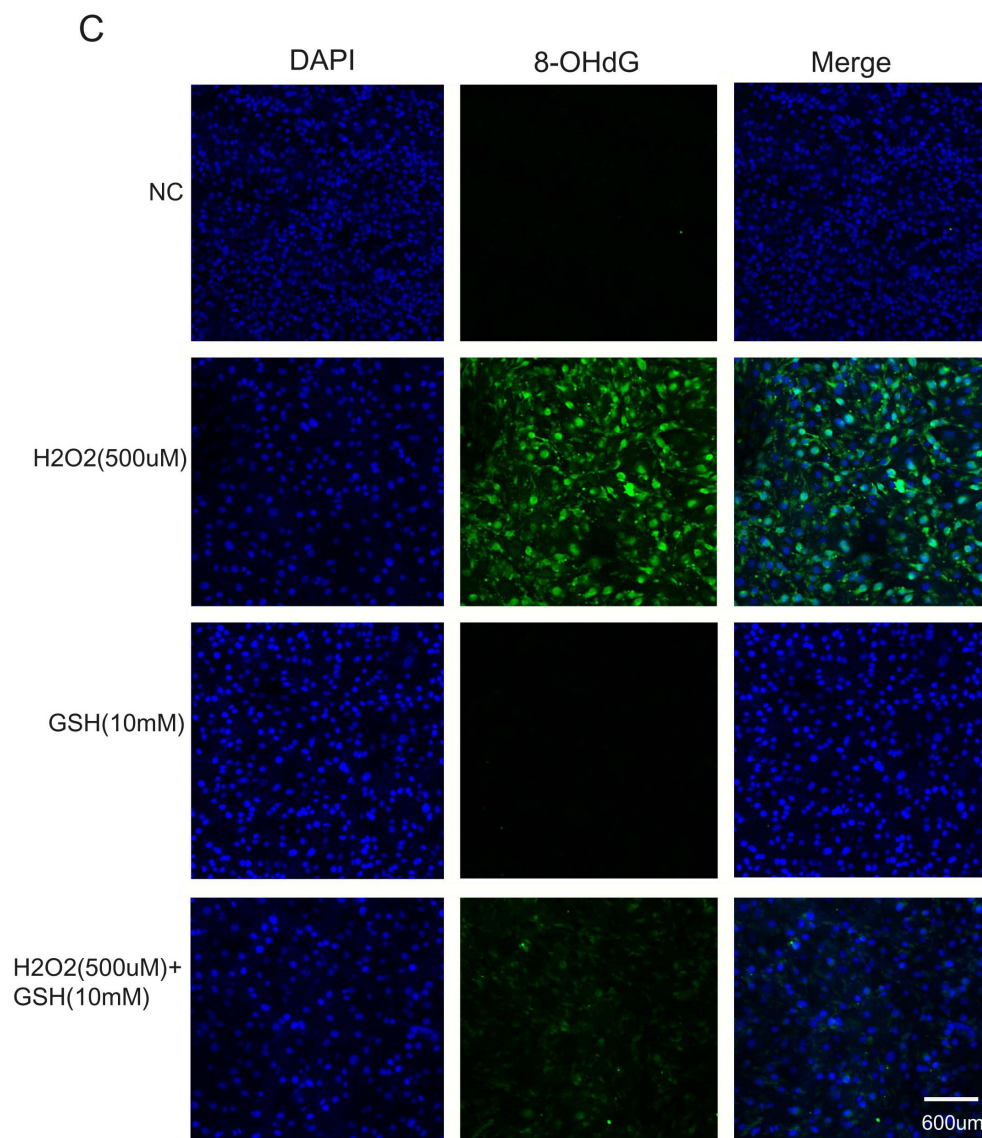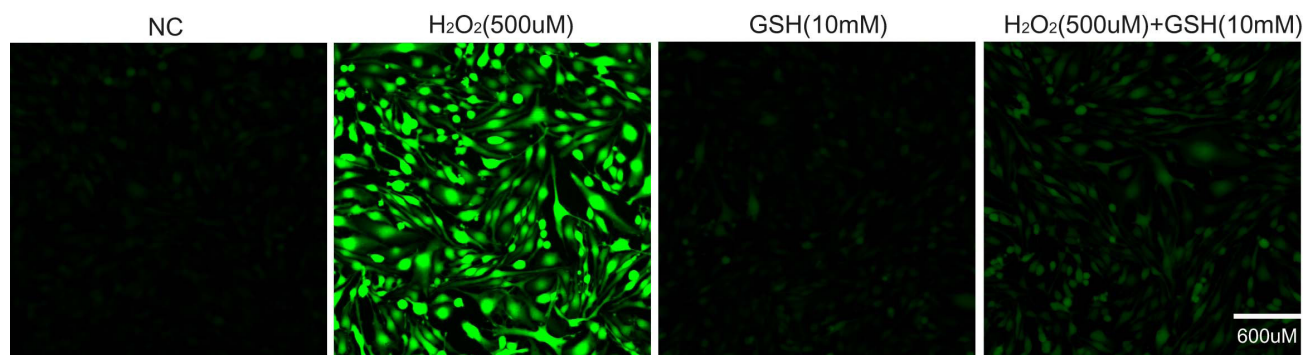
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Fig. 1. Continued.

Fig. 2. The effect of GSH on H_2O_2-induced ROS generation. H_2O_2 (500 μM) and/or GSH (10 mM) were treated in bEND.3 cells for 24 hr. ROS levels were measured using DCF-DA. ROS level in bEND.3 cells was increased in the H_2O_2 (500 μM) treatment group compared with normal control (NC) group. Also, ROS level was not change in the GSH (10 mM) treatment group compared with NC group. GSH decreased the H_2O_2-induced increase of DCF-DA-positive cells (green). Scale bar: 600 μm, 2′,7′-dichlorodihydrofluorescein diacetate (DCF-DA): green, 4′,6-diamidino-2-phenylindole (DAPI): blue.
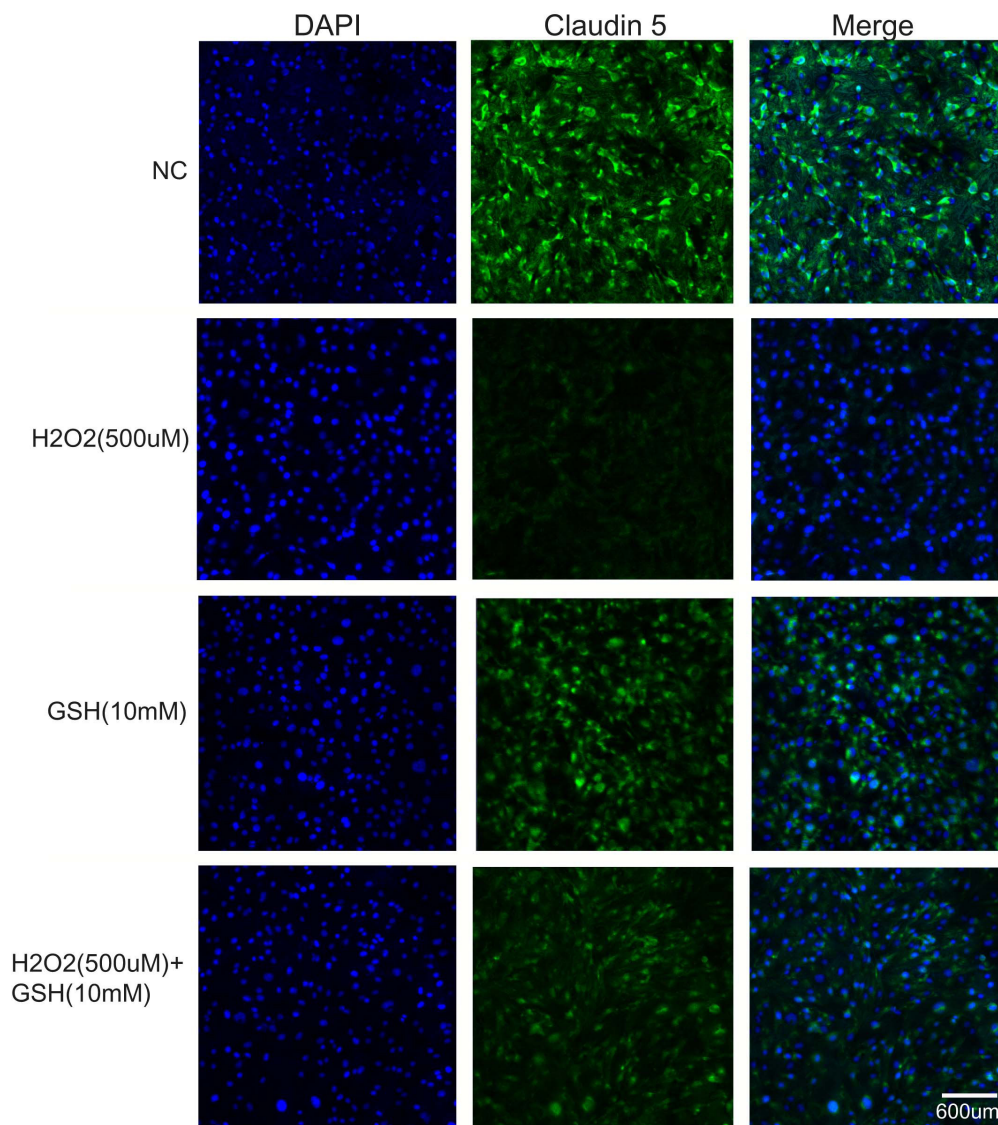
Fig. 3. The effect of GSH H_2O_2-induced decrease in tight junction proteins. H_2O_2 (500 μM) and/or GSH (10 mM) were treated in bEND.3 cells for 24 hr. The level of Claudin 5, a tight junction protein, was evaluated by immunocytochemistry. GSH attenuated H_2O_2-induced decrease in the number of Claudin 5-positive cells (green). Scale bar: 600 μm, Claudin 5: green, 4', 6-diamidino-2-phenylindole (DAPI): blue.

deletions, mutation, and mtDNA loss [45-47], our result suggests that GSH protects the mtDNA in brain capillary endothelial cell from H_2O_2-induced damages. We also showed that Claudin 5, a tight junction protein [48], is stabilized by GSH. Because the degradation of tight junction proteins is associated to the disruption of BBB and the progression of central nervous system diseases [49-52], our result suggests that GSH may protect BBB by inhibiting the degradation of tight junction proteins under oxidative stress. ROS regulates directly or indirectly a number of transcription factors [53]. Especially, ROS can promote the activation of Nrf2, which regulates antioxidant gene expression [53, 54]. Several studies demonstrated that Nrf2 activation inhibits

ROS generation [55] and NO generation [56] to protect cell damage against oxidative stress [22]. In addition, Nrf2 modulates the apoptosis and autophagy related signaling [23]. Nrf2 signaling is activated by the PI3K/Akt pathway and the ERK pathway to promote the expression of antioxidant genes during oxidative stress [57-61]. Specifically, Nrf2 is activated by phosphorylation of ERK [61-64]. Gunjima et al. demonstrated that SH-SY5Y cells were protected against oxidative stress through Nrf2-glutathione pathway [65]. However, the research on the protective effect of GSH through Nrf2/ERK pathway in brain capillary endothelial cells has not studied until now. In present study, we investigated the protective effect of GSH against oxidative stress in brain capillary
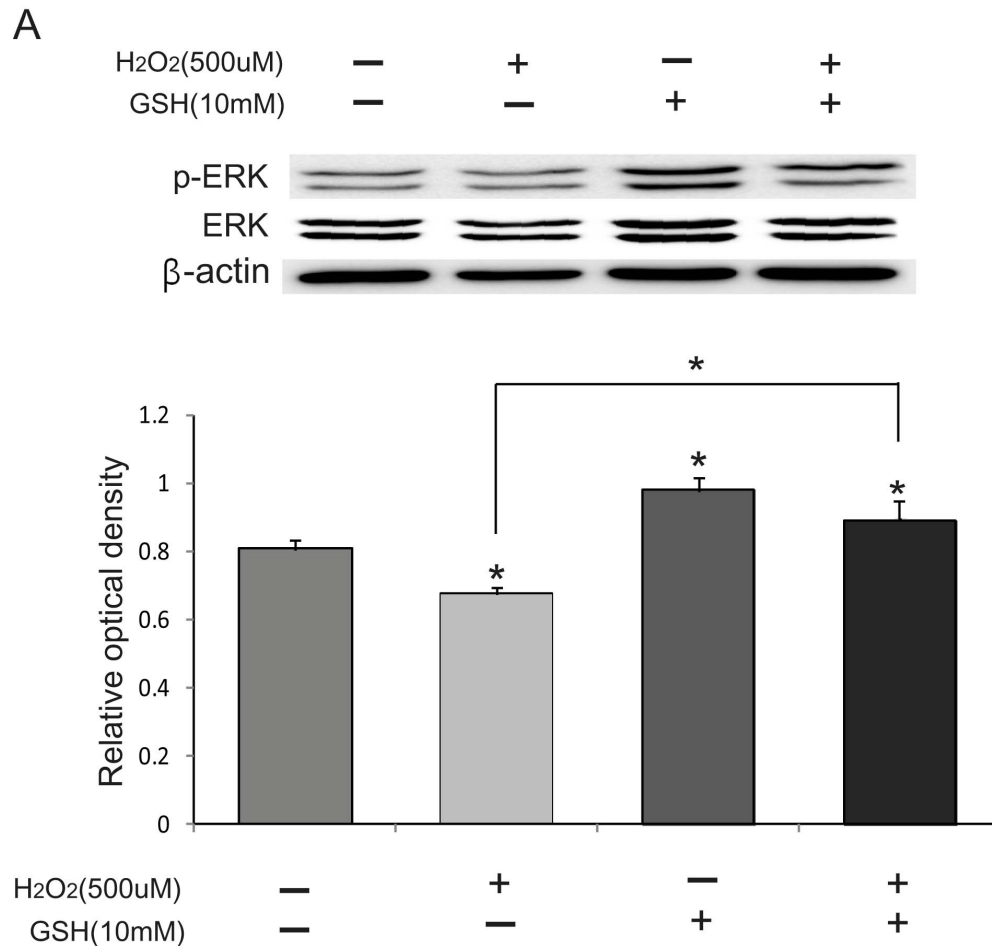
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Fig. 4. The effect of GSH on Nrf2 signaling. H_2O_2 (500 μM) and/or GSH (10 mM) were treated in bEND.3 cells for 24 hr. The protein level of Nrf2, ERK, phospho-ERK was evaluated by using Western blot analysis. (A) The phospho-ERK protein levels decreased by H_2O_2 treatment. GSH treatment increased the level of phospho-ERK. Bar graph showed the quantification of phosphor-ERK /ERK protein in all groups. (B) Nrf2 protein levels decreased by H_2O_2 treatment. GSH increased the basal level of Nrf2 proteins. GSH co-treatment increased Nrf2 protein levels compared to H_2O_2 treatment. β-actin was used as an internal control (mean±S.E.M., n=3) ([#]p<0.1, [*]p<0.05, [**]p<0.001).

endothelial cells. In addition, we showed that GSH may promote phosphorylation of ERK in brain capillary endothelial cells under oxidative stress (Fig. 4). Based on our results, we suggested that the protective mechanism of GSH may be related to the Nrf2/ERK pathway in brain capillary endothelial cells against oxidative stress. Several studies suggested that Nrf2 activates to protect the cells in early oxidative stress. However, in present study, we analyzed the expression of ERK and Nrf2 at late oxidative stress state (at 24 hrs after H_2O_2-induced oxidative injury). Even though the expression of ERK and Nrf2 were decreased in only H_2O_2 treatment group, GSH co-treatment group were protected brain capillary endothelial cells through activation of ERK and Nrf2 against oxidative stress. In addition, considering recent studies that excessive ROS inhibits the phosphorylation of ERK [66, 67], decreased ROS levels by GSH treatment in present study may

promote the phosphorylation of ERK and Nrf2 activation. In conclusion, we show that GSH protects brain capillary endothelial cells from H_2O_2-induced damages. GSH does this possibly by stabilizing tight junction proteins and activating Nrf2 signaling. Hence, this study suggests that GSH is a promising target to treat various central nervous system disorders and injuries characterized by oxidative stress.
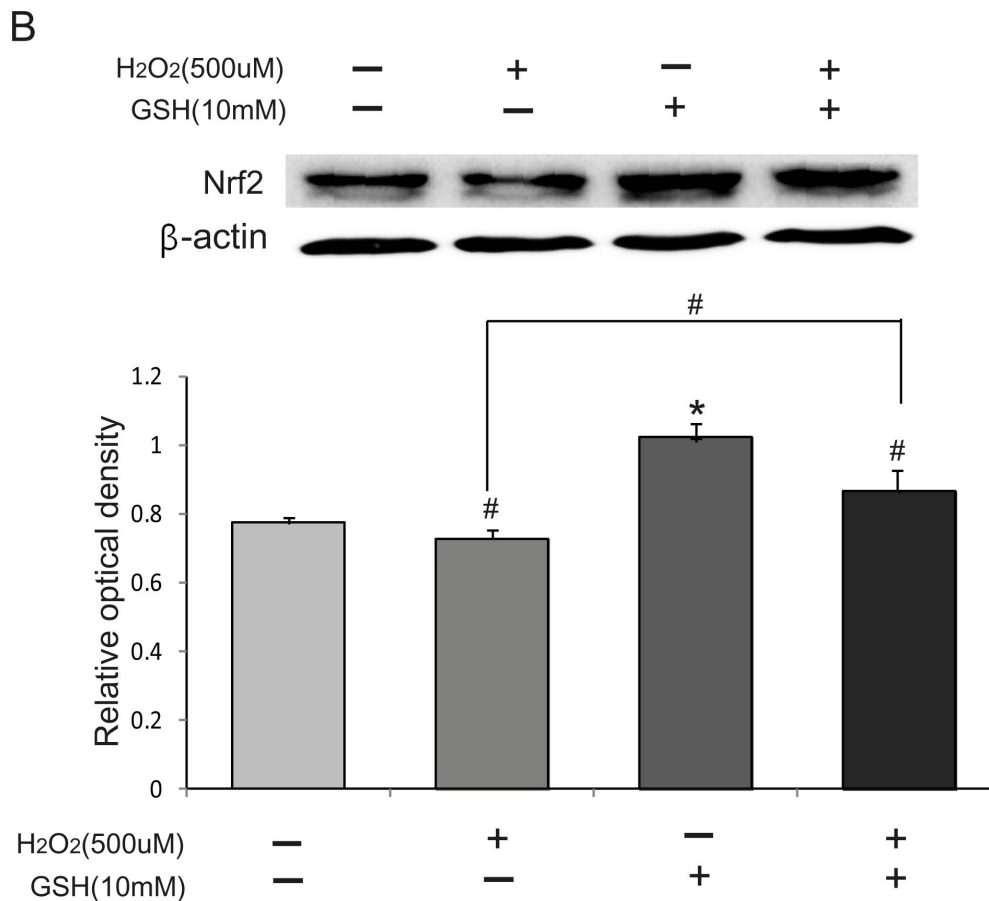
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Fig. 4. Continued.

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