RESEARCH ARTICLE

Changes in Nrf2 Expression in the Spinal Cord in Mouse Model of Experimental Autoimmune Encephalomyelitis

Sung Min Nam[1,§], Jun Ho Lee[2,§], Byung-Joon Chang[1], Jong-Hwan Lee[1,*] and Hyun-Jin Tae[3,*]

[1]Department of Anatomy, College of Veterinary Medicine, Konkuk University, Seoul 05030, Korea
[2]Institue of BioInnovation Research, Kolon Life Science, Inc., Magokdong-ro, Gangseo-gu, Seoul 07793, Korea
[3]Bio-Safety Research Institute, College of Veterinary Medicine, Chonbuk National University, Iksan 54596, Korea
*Corresponding author: hjtae@jbnu.ac.kr; jhlee21@konkuk.ac.kr

A B S T R A C T

In this study, we investigated the expression of nuclear factor erythroid-derived 2-related factor 2 (Nrf2) in the spinal cord using an experimental autoimmune encephalomyelitis (EAE) model. MOG_{35-55} was injected into female mice to induce an autoimmune response. Clinical scoring and a behavioral rotarod test were conducted to assess the successful modeling of EAE. Animals were sacrificed 1, 2, and 3 weeks after immunization. Spinal cords were prepared for morphometry, immunohistochemistry, and western blotting. Multifocal demyelination was observed in the white matter. Nrf2 immuno-reactivity was detected in astrocytes and neurons in spinal cord. Nrf2 immuno-reactive neurons and astrocytes had increased by 2 weeks after immunization, and their up-regulation was maintained until 3 weeks after immunization. Additionally, time-dependent up-regulation of Nrf2 in the spinal cord was investigated by western blotting. The level of NAD(P)H quinine oxidoreductase-1 protein, an Nrf2 target gene, was not changed during week 1, but showed a significant decrease 2 and 3 weeks after immunization. Parallel to up-regulated Nrf2, heme oxygenase-1, another target gene of Nrf2, was significantly augmented until 3 weeks after immunization. We conclude that Nrf2 expression in neurons is involved in an autoimmune attack in the spinal cord in an EAE model.

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INTRODUCTION

Multiple sclerosis (MS) is a demyelinating disease developed by an autoimmune attack on the central nervous system, characterized by destruction of myelin and multi-centric inflammation. It leads to serious motor disturbance and clinical disorders in MS range from relapsing-remitting to chronic-progressive form, associated with permanent neurological problems such as motor paralysis and ataxia (Tsujimura *et al.,* 2008; Dendrou *et al.,* 2015; Ransohoff *et al.,* 2015;).

Nuclear factor erythroid-derived 2-related factor 2 (Nrf2) is encoded by the NRE2LE gene and is a basic leucine-zipper transcription factor that regulates the expression of cytoprotective proteins including antioxidant enzymes (Kraft *et al.,* 2004). Nrf2 has been reported to mediate neuro-protection in Parkinson's

disease and cerebral ischemia (Shih *et al.,* 2005; Chen *et al.,* 2009) and to direct neuroprotection in the autoimmune neuro-inflammatory response in MS (Kraft *et al.,* 2004; Johnson *et al.,* 2010). Recent findings highlight the importance of Nrf2 in MS pathogenesis; in support of this, Nrf2 enhancement has been reported in the brains of various animal models of MS (Solis *et al.,* 2002; Kraft *et al.,* 2004; Johnson *et al.,* 2010; van Horssen *et al.,* 2010). In addition, Nrf2 was shown to be prominently increased in demyelinated lesions in the spinal cord of human MS patients (Licht-Mayer *et al.,* 2015).

However, previous studies using animal models of MS focused predominantly on Nrf2 expression in the brain. Lately, Nrf2 expression and its association with MS in the spinal cord of MS animal models are beginning to be reported (Linker *et al.,* 2011; Higashi *et al.,* 2017). In the present study, we attempted to investigate the expression of Nrf2 in a cellular subpopulation in the spinal cord using an experimental autoimmune encephalomyelitis (EAE) model,

§These authors contributed equally to this work.

which serves as an in vivo validation model for MS. Additionally, the EAE model in dogs is also a viable model for canine necrotizing encephalitis (Moon *et al.,* 2015). In the spinal cord, Nrf2 expression was studied using immunohistochemistry and western blotting 1, 2 and 3 weeks after immunization.

MATERIALS AND METHODS

Experimental animals and induction of EAE: Nine-week-old female C57BL/6 mice were achieved from the Hallym University (Chuncheon, South Korea). Animals were housed in a conventional state and allowed free access to food and water. The experimental procedures were adhered to the guidelines (NIH Guide for the Care and Use of Laboratory Animals, NIH Publication). EAE was induced according to the protocol of previous study (Lee *et al.,* 2014). Mice (n=14) were subcutaneously immunized with 200μg of myelin oligodendrocyte glycoprotein (MOG_{35-55}) peptide (AnaSpec; Fremont, CA, USA) which were emulsified in complete Freund's adjuvant (CFA) (H37Ra; Difco Laboratories, Detroit, MI, USA) supplemented with Mycobacterium tuberculosis (4 mg/ml, Sigma-Aldrich; St. Louis, MO, USA). Mice were intraperitoneally injected with pertussis toxin (200ng, Sigma-Aldrich) on day 0 and 2 after immunization. Only CFA was injected in the control mice.

Clinical scoring and evaluation of behavior: Animals were daily checked for clinical scores with the following criteria - 0. healthy; 1. flaccid tail; 2. hind limb mild paralysis; 3. paralysis of both hind legs with residual mobility; 3.5. complete paralysis of both hind legs; 4. complete paralysis of both hind legs with front limb paralysis; 5. moribund or dead (Pöllinger *et al.,* 2009). The motor performance of EAE mice was assessed using rotarod (Med Associates, Inc., St Albans, VT, USA) by checking the latency time to fall (Tsunoda and Fujinami, 2010).

Histology: Mice (n=7) were deeply anesthetized by isoflurane (3% in oxygen), perfused trans-cardially with PBS and 10% neural buffered formaldehyde (NBF). The spinal cord was dissected and post-fixed with 10% NBF. Tissues were paraffin-embedded and sectioned at 6 μm with microtome. First, sections were stained with Luxol Fast Blue (LFB) (Sigma-Aldrich, St. Louis, MO, USA) to assess demyelination.

For immunohistochemistry (IHC) and double immunofluorescence staining, sections were processed according to the previous study (Chang *et al.,* 2012). Briefly, sections were incubated (overnight at 4°C) with rabbit anti-Nrf2 monoclonal antibody (1:200, NOVUS Biologicals, Littleton, CO, USA), and biotinylated-conjugated anti-rabbit antibody, horseradish peroxidase-conjugated streptavidin (1 hr at room temperature). IHC sections were visualized by diaminobenzidine tetrachloride (Vector Lab, Burlingame, CA, USA) and contrasted with hematoxylin counterstain. For double staining, sections were incubated with anti-Nrf2 monoclonal antibody (1:100), mouse anti-glial fibrillary acidic protein (GFAP) (1:200, Chemicon International, Temecula, CA), and mixture of both FITC-conjugated goat anti-mouse IgG (1:200, Jackson ImmunoResearch,

West Grove, PA) and Cy3-conjugated goat anti-rabbit IgG (1:200, Jackson ImmunoResearch). Counterstain was conducted with 4,6-diamidino 2-phenylindole (DAPI; Vector Lab) for nucleus. Images were taken using digital image analyzer (MCID; Inter-Focus Imaging Ltd., Cambridge, UK) and confocal microscope (LSM510 META NLO, Carl Zeiss, Germany). Numbers of Nrf2 immuno-reactivity positive ($^+$) cells were counted in the spinal cord.

Western blotting: Mice (n=7) in each group were sacrificed and spinal cords were dissected and stored at -80°C until analysis. Tissues were processed according to the previous study (Chang *et al.,* 2012). After blotting, membranes were blocked with skim milk and incubated with Nrf-2 antibody and other antibodies (NQO1, HO1, Actin and secondary HRP-conjugated anti-IgG). Immuno-reactive proteins were detected using ECLplus Western Blot Detection Kit (GE Healthcare, Piscataway, NJ, USA). Bands density was quantitated using a density-metric Multi Gauge program (Fujifilm Corporation, Tokyo, Japan).

Statistical analysis: Data was analyzed using ANOVA followed by Duncan's multiple rage as *post hoc* comparison test with SAS (SAS Institute Inc., version 9.2, Cary, NC, USA). A value of significant threshold was set as $P<0.01$. The analysis of motor performance of behavioral test adopted a Kruskal-Wallis nonparametric one-way analysis of variance and followed by a two-tailed Mann-Whitney U test. Statistically, a value of $P<0.05$ was significant.

RESULTS

Clinical and behavior scores in EAE model: Clinical scores of EAE induction were initially recorded at day 11 after MOG_{35-55} immunization, and clinical signs progressed rapidly. In most mice in the experimental group, clinical scores were graded "5" 3 weeks after MOG_{35-55} immunization (Fig. 1A). The staying period on the rotarod in the MOG_{35-55} immunization group was significantly shorter than that of the control group. The latency time of immunized mice declined significantly beginning 1 week after MOG_{35-55} immunization. After 3 weeks, none of the mice in the MOG_{35-55} immunization group were able to remain on the rotating drum (Fig. 1B).

LFB staining and measurement of demyelination: Areas of delamination appeared in a multifocal fashion in the white matter of spinal cords of animals in the MOG_{35-55} immunization group, while this phenomenon was not observed in the control group (Figs. 2A-F). The delamination areas showed significant time-dependent increases after MOG_{35-55} immunization (Fig. 2G).

Nrf2 IHC in spinal cord: In the control group, Nrf2 immunoreactivity was detected in the grey and white matter of the spinal cord. The numbers of observed $Nrf2^+$ cells in the spinal cord were similar to the control and 1 week after MOG_{35-55} injection. Thereafter, the number of $Nrf2^+$ cells in the spinal cord increased significantly compared to the controls 2 and 3 weeks after MOG_{35-55} injection. Subsequently, 3 weeks after MOG_{35-55} injection, the number of $Nrf2^+$ cells in grey matter had decreased compared to that at 2 weeks (Fig. 3).
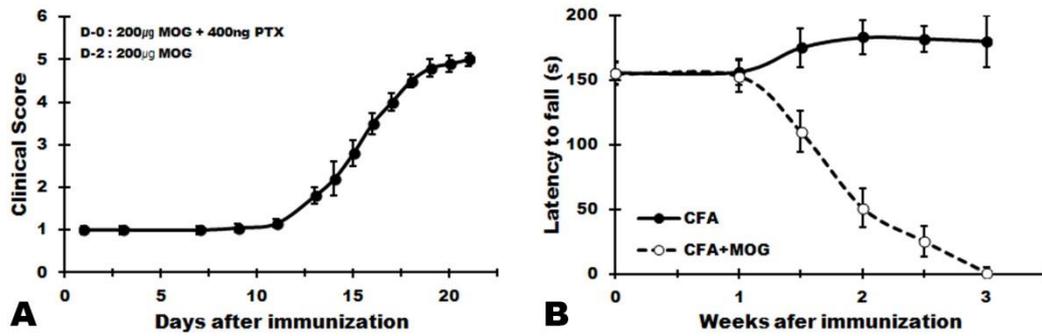
Fig. 1: Clinical score (A) and behavioral test (B). Clinical score was significantly increased on 11th day, after the induced immunization (MOG$_{35-55}$ peptide injection), most of the mice clinical score were "5" on 3rd week (A), results shown the daily mean clinical scores (n=21). Immunized mice latency time (second) was significantly declined after 1 week on rotarod test, the mice could not maintain on rotating drum after 3 weeks (B). Data are expressed as the mean ± S.E.M., and representative of experiments repeated three times.
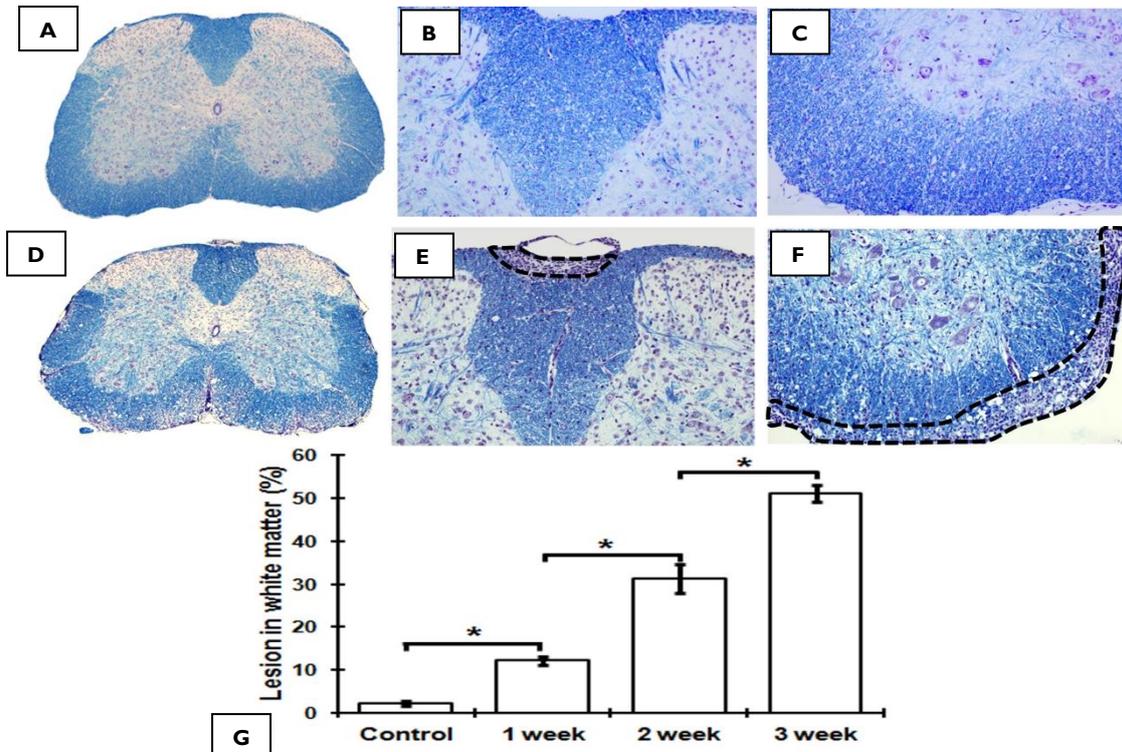
Fig. 2: Detection of demyelination on the spinal cord with LFB staining (A-F). Demyelination has not been shown by the control group (A; x 100; B and C; x 200). However, MOG$_{35-55}$ peptide treatment group's demyelination was significantly increased with time dependently at 3 week (D; x 100; E and F; x 200). Demyelination area in the spinal cord is outlined by the dashed black line. Demyelination data was present with graph (G). Data are expressed as the mean±S.E.M. and representative, ANOVA, P<0.01, Duncan test. Meanwhile, demyelination was not detected in the gray matter of spinal cord.

Double immunofluorescence staining: Double staining was performed to differentiate the type of cells in the spinal cord expressing Nrf2 (Fig. 3). Large numbers of astrocyte in the white matter expressed Nrf2, while some neurons in grey matter showed Nrf2 positivity. We confirmed that, in the spinal cord, the Nrf2 expressing cells in white matter were astrocytes, while in the grey matter, Nrf2 was expressed in neurons (Fig. 3).

Western blot analysis: The results of western blotting revealed a pattern of Nrf2, HO1, and NQO1 protein expression in the spinal cord at 1, 2 and 3 weeks after MOG$_{35-55}$ immunization. As EAE progressed, the level of Nrf2 was significantly upregulated compared to the control at 2 and 3 weeks after immunization and peaking at 2 weeks. We also detected time-dependent changes in the Nrf2 target gene HO1, with increases during weeks 2 and 3 after immunization. In contrast, another Nrf2 target gene, NQO1, remained unchanged 1 week after immunization and significantly decreased 2 and 3 weeks after MOG$_{35-55}$ immunization (Fig. 4).

DISCUSSION

This study was designed to investigate the morphometry of demyelination and the expression of Nrf2 in the EAE mouse spinal cord. LFB staining demonstrated multifocal time-dependent demyelination in the white matter of the spinal cord. Nrf2 upregulation was detected in astrocytes in the white matter and in neurons in the grey matter. The numbers of Nrf2+ astrocytes and neurons increased 2 weeks after MOG$_{35-55}$ immunization, and then decreased at 3 weeks. The protein expression levels of Nrf2 measured by western blotting were similar to the immuno-histochemistry results, showing a peak at 2 weeks and a subsequent decrease 3 weeks post-MOG$_{35-55}$ immunization.
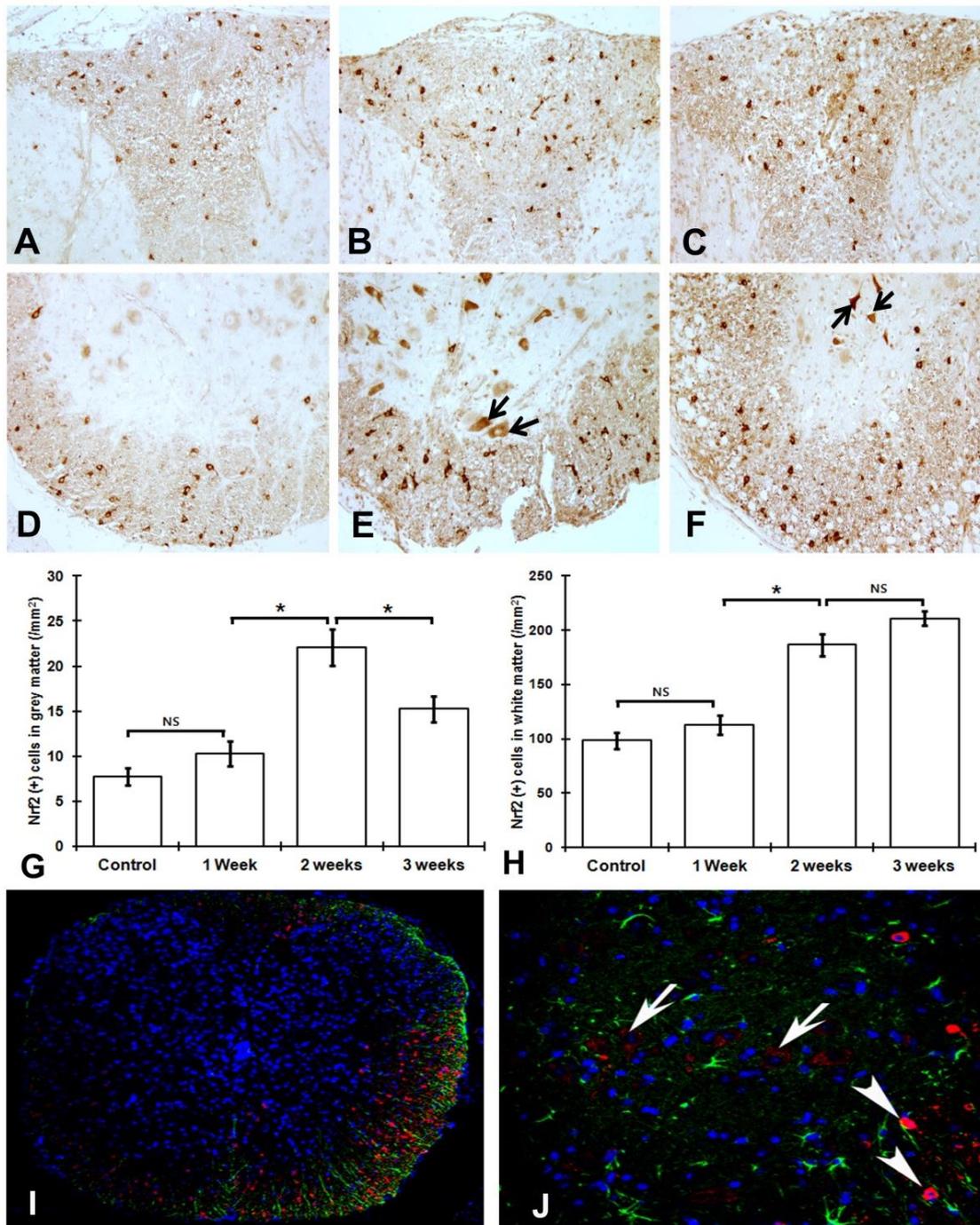
Fig. 3: Immnohistochemistry of Nrf2 in the spinal cord (A-F) and the positive cell number in gray matter (G) and white matter (H). Control (A and D), 2 weeks (B and E) and 3 weeks (C and F) after MOG$_{35-55}$ peptide treatment. Data are expressed as the mean±S.E.M. and representative, ANOVA, P<0.01, Duncan's test. Arrows are marked neurons of Nrf2 immuno-reactivity. A-F: x 200. Double immunofluorescence staining of Nrf2 (red), astroyctes (GFAP, green) and neurons (blue) 3 week after immunizations (I x 100, J x 200). Nrf2 and astrocyte co-location was detected in white matter, and co-location with neuron was detected in grey matter of spinal cord. Nrf2 expressed cell is astrocyte in white matter and is neuron in grey matter of spinal cord. Arrow indicated Nrf2 expressed neurons in the grey matter, arrow head indicated Nrf2 expressed astrocytes in white matter.

Experimentally, MOG$_{35-55}$ immunization is a convenient animal model of EAE, which may correspond to human MS (Lee *et al.*, 2014). In this study, clinical grading scores increased 2 weeks after MOG$_{35-55}$ immunization, and behavioral disorders were recorded after 1 week. In the spinal cord, demyelinated white matter was observed 1 week after MOG$_{35-55}$ immunization, and demyelination progress paralleled the clinical symptoms and behavioral disorders. These phenomena were reported in our previous study and are known to be typical characteristics of MS (Lee *et al.*, 2014).

An inflammatory response and oxidative stress are detected in MS, and Nrf2 has been reported as being closely related to these inflammatory and oxidative stresses in the brain of MS patients (Gilgun-Sherki *et al.*, 2004; Pöllinger *et al.*, 2009; Johnson *et al.*, 2010; Rosenbaum *et al.*, 2012). In our EAE-modeled spinal cords, increased Nrf2 protein levels were again confirmed. In double-stained tissue, the Nrf2^{+} cells were astrocytes in the white matter and neurons in the grey matter. Nrf2^{+} neurons have been reported in the brains of EAE animal model (Linker *et al.*, 2011).
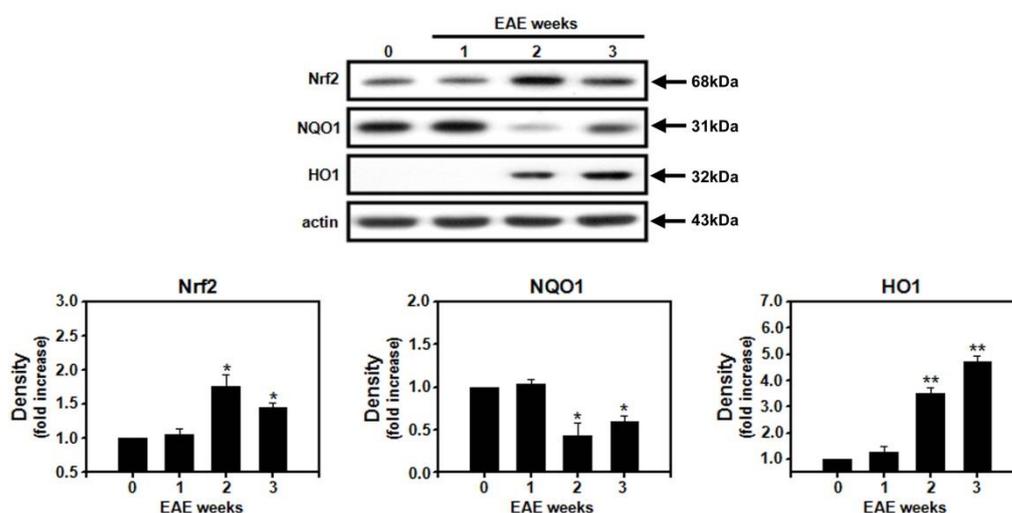
Fig. 4: Expression of Nrf2, NQO-1 and HO1 in the spinal cord. Total levels of Nrf2 and HO1 were increased from week 2 post-immunization of MOG$_{35-55}$, however, the level of NQO-1 decreased on 2 and 3 weeks. Results shown were mean±S.E.M and representative of experiments repeated three times. Each group compare with ANOVA, P<0.01. Duncan's test.

Additionally, the activation of Nrf2-ARE pathway was detected in the spinal cord of amyotrophic lateral sclerosis (ALS) model mice (Kraft *et al.*, 2007). Furthermore, Vargas *et al.* (2005) reported that the astrocytes had strong Nrf2 expression and enhanced immune response in spinal cord.

The present results confirmed that, within 3 weeks of immunization, expression of Nrf2 is detectable in the neurons and astrocytes in the grey and white matter, respectively, of the spinal cord of EAE mice. Neurons also showed expression of Nrf2 in the grey matter of spinal cord, where the number of Nrf2$^+$ cells was augmented at 2 and 3 weeks after immunizations in this study. Recently, Linker *et al.* reported that Nrf2 was expressed in the neurons and astrocytes of the spinal cord 41 and 74 days after EAE modeling, respectively, (Linker *et al.*, 2011). Accordingly, Nrf2 was reported to be expressed in the astrocytes and macrophages of spinal cord lesions in MS patients (van Horssen *et al.*, 2010). However, Nrf2 was also detected in the oligodendrocytes at the initial demyelination sites in MS patients (Licht-Mayer *et al.*, 2015). Interestingly, the number of Nrf2$^+$ neurons paralleled the level of Nrf2 protein determined by western analysis, and grey matter of the spinal cord was unaffected by the inflammatory and oxidative attack of MOG$_{35-55}$ immunizations. This suggests that Nrf2 expression in neurons is more closely related to the impact on the protein level of Nrf2 in the spinal cord after EAE. Additionally, Johnson *et al.* (2010) reported that Nrf2 knockout exacerbated the severity of EAE, indicating that Nrf2 may participate in delaying the development of autoimmune disease.

To clearly define the meaning of Nrf2 upregulation in the previous and present studies, we focused on the fact that Nrf2 is an oxidative stress response mechanism initiating the expression of cytoprotective proteins. For instance, targets of Nrf2 include NQO1, glutamate-cysteine ligase catalytic (Gclc), HO1, glutathione S-transferase (GST), UDP-glucuronosyltransferase (UGT), and multi drug resistance-associated proteins (Mrps) (Solis *et al.*, 2002; Maher *et al.*, 2007; Wang and Dore,

2007; Yamamoto *et al.*, 2008; Jarmi and Agarwal, 2009; Zhao *et al.*, 2009).

The protein level of Nrf2 was upregulated in spinal cord tissue in the EAE model of MS and paralleled enhanced expression of the HO1 protein in the present study. Similarly, ginsenoside Rb1-induced protection of neural progenitors from oxidative stresses was mediated by upregulation of Nrf2 and its target HO1 (Ni *et al.*, 2014). The protective effects of HO1 are associated with attenuation of excessive nitric oxide production and cellular toxicity. Several earlier reports revealed HO1 involvement in acute lung injury sepsis, atherosclerosis, hypertension, and kidney injury (Yamamoto *et al.*, 2008; Jarmi and Agarwal, 2009). Our finding that HO1 is induced in the spinal cord in the EAE model indicates concomitant induction of HO1 in the Nrf2-dependent cytoprotective mechanism. NQO1 is another target gene of Nrf2 (Wang *et al.*, 2007) and catalyzes the reduction of quinines while preventing their participation in redox cycling. NQO1 is a broad-spectrum antioxidant that maintains antioxidative alpha-tocopherol and coenzyme Q10 in their active states by scavenging superoxide (Kraft *et al.*, 2004; Wang *et al.*, 2007). During autoimmune demyelination, NQO1 undergoes time-dependent changes different from Nrf2 and HO1 expression. In the present study, the NQO1 level in the spinal cord was significantly decreased 2 and 3 weeks after immunization. However, NQO1 expression was increased in the brain in the EAE animal model (Li *et al.*, 2013). Along with Nrf2-HO1, NQO1 was increased in the brain as a protective mechanism against lead toxicity (Ye *et al.*, 2016). To the contrary, Agúndez *et al.* (2014) reported that the NQO1 gene does not show a significant correlation in comparison between MS patients and healthy controls. These inconsistent NQO1 results suggest the possibility of site-dependent differences in NQO1 expression or Nrf2-independent changes in NQO1 expression in the spinal cord. However, clarifying the correlation of NQO1 with Nrf2 in the spinal cord in this model will require additional intensive studies.

Conclusions: The present results indicate the involvement of Nrf2 signaling in regulation of cytoprotective genes in response to inflammatory mediators in MS pathology in spinal cords. Along with the induction of Nrf2, there are multidirectional changes in the expression of potent target genes including HO1 and NQO1. Expressional change of Nrf2 in the spinal cord of the EAE model suggests that future investigations in multiple sclerosis patients focusing on this molecule will open a window to increase understanding of the pathology of this disease.

Authors contribution: HJ Tae, SM Nam, JH Lee and JH Lee conceived and conducted experiments, and collected the data. HJ Tae, SM Nam, JH Lee and JH Lee analyzed the data and wrote the manuscript. BJ Chang participated in designing and discussing the study. All authors approved the final version of the manuscript.

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