

Cellular and regional specific changes in multidrug efflux transporter expression during recovery of vasogenic edema in the rat hippocampus and piriform cortex

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In the present study, we investigated the characteristics of drug efflux transporter expressions following status epilepticus (SE). In the hippocampus and piriform cortex (PC), vasogenic edema peaked 3-4 days after SE. The expression of breast cancer resistance protein (BCRP), multidrug resistance protein-4 (MRP4), and p-glycoprotein (p-GP) were decreased 4 days after SE when vasogenic edema was peaked, but subsequently increased 4 weeks after SE. Multidrug resistance protein-1 (MRP1) expression gradually decreased in endothelial cells until 4 weeks after SE. These findings indicate that SE-induced vasogenic edema formation transiently reduced drug efflux pump expressions in endothelial cells. Subsequently, during recovery of vasogenic edema drug efflux pump expressions were differentially up-regulated in astrocytes, neuropils, and endothelial cells. Therefore, we suggest that vasogenic edema formation may be a risk factor in pharmacoresistent epilepsy. [BMB Reports 2015; 48(6): 348-353]

INTRODUCTION

Up to 40% of epileptic patients show refractory seizure activity that cannot be controlled by conventional antiepileptic drugs (AEDs) (1). Since the ATP binding cassette (ABC) family is a major contributor in the exclusion of drugs from the brain as drug efflux transporters, ABC family expression is considered an important factor in the therapeutic failure of AEDs (2). The p-glycoproteins (p-GP, ABCB1, MDR1), breast cancer resistance proteins (BCRP, ABCG2), multidrug resistance protein-1s (MRP1, ABCC1), and multidrug resistance protein-4s

(MRP4, ABCC4) are well-characterized ABC family members that function as drug efflux transporters across the blood-brain barrier (BBB) and in parenchymal cells such as astrocytes (3-8). Similar to the BBB, ABC family in parenchymal cells acts as a second drug barrier or transporter to drug permeability in the brain (9). Briefly, p-GP in brain parenchyma prevents entry of pharmacological agents (9). BCRP transports solutes out of brain parenchyma across BBB (10). MRP1 regulates intracellular redox potential, flux of ions (11), and elimination of endo- and xenobiotics (12). MRP4 is involved in nucleotide/nucleoside transport *in vivo* (13).

In the human patients with refractory epilepsy, the ABC family was highly expressed not only in vascular endothelial cells, but also in brain parenchymal cells (14). This hypothesis is supported by experimental studies demonstrating that prolonged seizure activity in rodents increases p-GP expression, which correlates with decreased brain levels of some AEDs (8, 15). Zhang *et al.* (16) reported that p-GP expression is increased in astrocytes and blood vessels within the hippocampus 2 weeks after status epilepticus (SE). In female rats, p-GP expression in brain capillary endothelial cells is increased 48 h after SE, in the hippocampus, but not in the cortex (17). In contrast, Kuteykin-Teplyakov *et al.* (18) reported that between 6 and 24 h after onset of SE, genes encoding p-GP, MRP1, and MRP5 are downregulated in the hippocampus, amygdala, and piriform cortex (PC). This initial decrease in expression is followed by normalization and then increased expression, which reaches a maximum 2 days after SE. Therefore, the temporal and regional alterations of different efflux transporter expression after SE are still unclear.

SE causes vasogenic edema from increases in pinocytosis at the level of the cerebral endothelium (19) and permeability of the BBB (20). Since the ABC family is expressed in the BBB (6-8), it is likely that alterations in drug efflux transporter expression may be one of the undesirable consequences of BBB modification after vasogenic edema. Therefore, we investigated the correlation between vasogenic edema formation and changed drug efflux pump expressions to understand the mechanism of seizure-induced, over-expression of drug efflux transporters.

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RESULTS

ABC family expressions in the hippocampus following SE

Fig. 1 shows that serum extravasation was detected in the rat hippocampus 12 h after SE ($P < 0.05$ vs. non-SE animals; Fig. 1A and 1B). SE-induced serum extravasation peaked 3-4 days after SE ($P < 0.05$ vs. non-SE animals) and was maintained until 4 weeks after SE. BCRP expression was decreased at 12 h-1 day after SE ($P < 0.05$ vs. non-SE animals; Fig. 2A and 2B) and recovered to non-SE levels 3 days after SE (Fig. 2A and 2B). Four days to 4 weeks after SE, BCRP expression was increased, as compared to non-SE animals ($P < 0.05$ vs. non-SE animals; Fig. 2A and 2B). MRP1 expression gradually decreased from 4 days to 4 weeks after SE ($P < 0.05$ vs. non-SE animals; Fig. 1A and 1B). MRP4 expression was significantly reduced 12 h-3 days after SE ($P < 0.05$ vs. non-SE animals; Fig. 1A and 1B) and recovered to non-SE level 4 days to 1 week after SE. Four weeks after SE, MRP4 expression was increased to 1.51-fold of non-SE animals ($P < 0.05$ vs. non-SE animals; Fig. 1A and 1B). p-GP expression decreased for 4 days after SE ($P < 0.05$ vs. non-SE animals; Fig. 1A and 1B) normalized to basal level at 1 week after SE, and increased to 1.6-fold of non-SE animals at 4 weeks after SE ($P < 0.05$ vs. non-SE animals; Fig. 1A and 1B).

ABC family expressions in the PC following SE

Similar to the hippocampus, serum extravasation was detected in the rat PC 12 h after SE ($P < 0.05$ vs. non-SE animals; Fig. 2A and 2B). SE-induced serum extravasation peaked 3-4 days after SE ($P < 0.05$ vs. non-SE animals) and was maintained until 4 weeks after SE. BCRP expression was decreased at 12 h-1 day after SE ($P < 0.05$ vs. non-SE animals; Fig. 2A and 2B) and recovered to non-SE levels 3 days after SE (Fig. 2A and 2B). Four days to 4 weeks after SE, BCRP expression was increased, as compared to non-SE animals ($P < 0.05$ vs. non-SE animals; Fig. 2A and 2B). MRP1 expression gradually decreased from 12 h to 4 weeks after SE. MRP1 expression was 0.64-fold of non-SE animals 4 weeks after SE ($P < 0.05$ vs. non-SE animals; Fig. 2A and 2B). MRP4 expression was significantly reduced 1-4 days after SE ($P < 0.05$ vs. non-SE animals; Fig. 2A and 2B). MRP4 expression was increased to 1.2-fold of non-SE animals 1-4 weeks after SE ($P < 0.05$ vs. non-SE animals; Fig. 2A and 2B). p-GP expression was decreased 3 days to 1 week after SE ($P < 0.05$ vs. non-SE animals; Fig. 2A and 2B). Four weeks after SE, p-GP expression was increased to 1.2-fold of non-SE animals ($P < 0.05$ vs. non-SE animals; Fig. 2A and 2B).

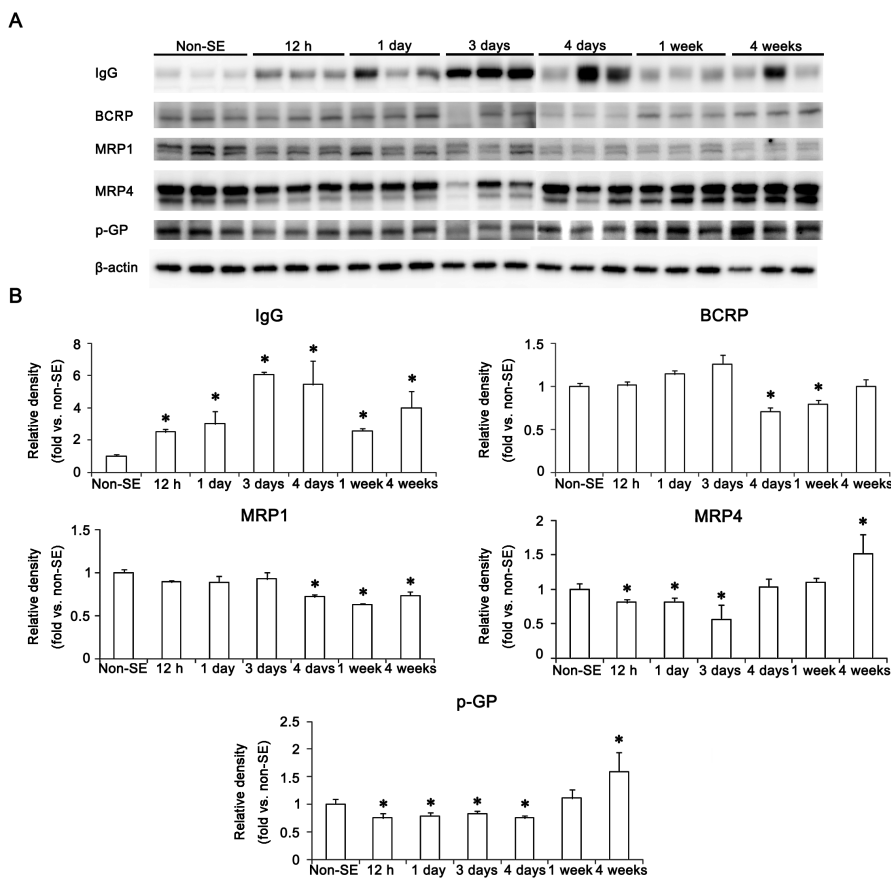


Fig. 1. Protein expression levels of IgG (heavy chain) and ABC family in the hippocampus following SE. (A) Western blot images of IgG and ABC family in the hippocampus. IgG level is peaked 3-4 days after SE. BCRP expression is decreased 4 days after SE, and recovered at 4 weeks after SE. MRP1 expression is gradually decreased 4 days-4 weeks after SE. MRP4 expression is reduced 12 h-3 days after SE, and recovered 4 days-1 week after SE, and increased 4 weeks after SE. p-GP expression is decreased until 4 days after SE, normalized to basal level 1 week after SE, and increased 4 weeks after SE. (B) Quantitative values (mean \pm S.E.M) of IgG, BCRP, MRP1, MRP4, and p-GP in the hippocampus, based on Western blotting ($n = 3$ per each group). Significant differences from non-SE animals, * $P < 0.05$.

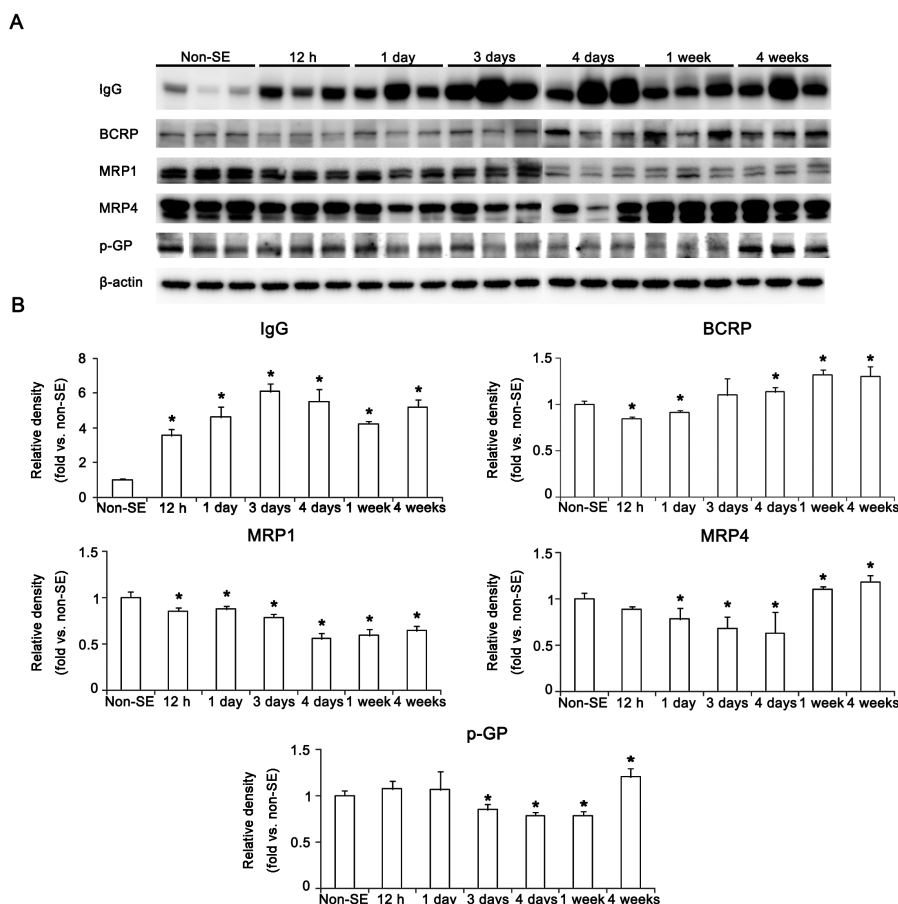


Fig. 2. Protein expression levels of IgG (heavy chain) and ABC family in the PC following SE. (A) Western blot images of IgG and ABC family in the PC. IgG level is peaked 3-4 days after SE. BCRP expression is decreased 12 h-1 day after SE, recovered 3 days after SE, and increased 4 days-4 weeks after SE. MRP1 expression is gradually decreased from 12 h to 4 weeks after SE. MRP4 expression is reduced 1-4 days after SE and increased 1-4 weeks after SE. p-GP expression is decreased 3 days-1 week after SE, and increased 4 weeks after SE. (B) Quantitative values (mean \pm S.E.M) of IgG, BCRP, MRP1, MRP4, and p-GP in the PC, based on Western blotting (n = 3 per each group). Significant differences from non-SE animals, *P < 0.05.

Upregulated BCRP expression in reactive astrocytes following SE

In non-SE animals, BCRP expression was detected in astrocytes, but not in endothelial cells in the hippocampus and the PC (Fig. 3A). Four weeks after SE, BCRP expression in the hippocampus was increased in some reactive astrocytes and was recovered to non-SE level (data not shown). In the PC, BCRP expression was increased in palisade astrocytes around vasogenic edema lesion (Fig. 3B).

Reduced MRP1 expression in endothelial cells following SE

In non-SE animals, MRP1 expression was observed in endothelial cells, but not in astrocytes, within the hippocampus and the PC. Following SE, MRP1 expression disappeared in endothelial cells in both regions (data not shown).

Altered MRP4 expression from endothelial cells to neuropils following SE

In non-SE animals, MRP4 expression was detected only in endothelial cells within the hippocampus and the PC (Fig. 3C). Four weeks after SE, MRP4 expression was observed in neuro-

pils within the hippocampus and the PC (Fig. 3D).

Cellular specific alteration of p-GP expression between the hippocampus and the PC following SE

In the hippocampus and the PC in non-SE animals, p-GP expression was observed in endothelial cells, not in astrocytes (Fig. 4A and 4C). Four weeks after SE, p-GP expression was observed in reactive astrocytes, but not in endothelial cells (Fig. 4B). In the PC, p-GP expression was observed in endothelial cells, but not in palisade astrocytes (Fig. 4D).

DISCUSSION

Basically, the ABC family is predominantly localized in the luminal membrane of endothelial cells (9). Interestingly, p-GP is overexpressed in both reactive astrocytes (14, 16, 21) and endothelial cells of the BBB (22). Since the endothelial BBB may be disrupted in seizures, overexpressed p-GP in astrocyte foot processes extending onto capillaries, as seen in epileptic patients, was suggested to act as a second barrier in BBB function (14). Therefore, we hypothesized that BBB disruption would

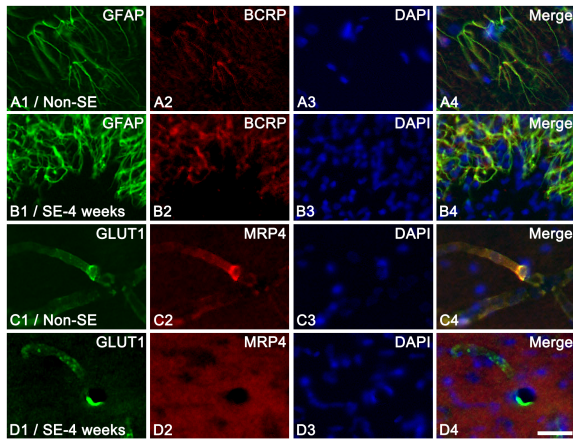


Fig. 3. Double immunofluorescence of GFAP/GLUT1 and BCRP/MRP4 following SE. (A) In control animals, BCRP expression is observed in astrocytes in the PC in non-SE animals. (B) Four weeks after SE, BCRP expression is increased in palisade astrocytes around vasogenic edema lesions within the PC. (C) In non-SE animals, MRP4 expression is detected only in endothelial cells. (D) Four weeks after SE, MRP4 expression is observed in neuropil within the hippocampus. Bar = 12.5 μ m.

affect ABC family expression following SE. As expected, serum extravasation preceded or coincided with down-regulation of ABC family expression in the hippocampus and the PC. Since the ABC family is one of the barrier functional components in the BBB (9), our findings point to the down-regulation of ABC family expression as one consequence of endothelial or astroglial damage during vasogenic edema formation.

In the present study, BCRP, MRP4, and p-GP expressions increased in the PC 4 weeks after SE. In our previous studies (23-26), the volume of vasogenic edema lesion peaked 3-4 days after SE, and then recovered 1-4 weeks after SE. The recovery of vasogenic edema lesion is accompanied by a progressive increase in vascularization (27), BBB repair and/or the formation of palisade astroglial barrier around vasogenic edema core (24, 26, 28). Therefore, it is likely that neovascularization, reactive astrogliosis, and/or BBB repair may be one of the factors to up-regulate ABC family expression during vasogenic edema formation. Unexpectedly, the biphasic alteration in ABC family expression showed cellular/regional specific patterns in the present study. In the hippocampus, both BCRP and p-GP expression were increased in some reactive astrocytes, while MRP4 expression was observed in neuropils. In the PC, BCRP expression was increased in palisade astrocytes around vasogenic edema lesions. Similar to the hippocampus, MRP4 expression was observed in neuropils. However, p-GP expression was observed in endothelial cells, not in palisade astrocytes. These cellular/regional discrepancies may be a consequence of the severity of vasogenic edema formation. Vasogenic edema formation and astroglial loss in the PC is

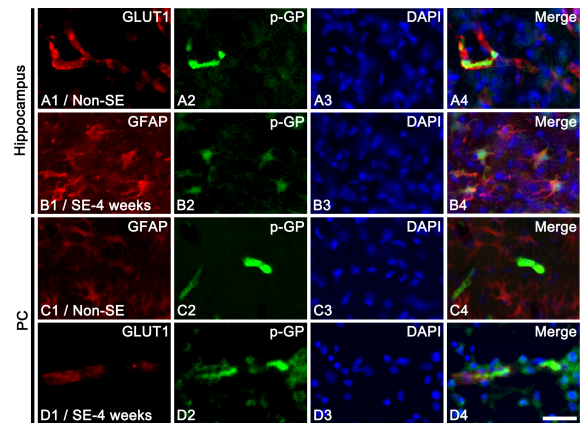


Fig. 4. Double immunofluorescence of GLUT1/GFAP and p-GP following SE. (A) In non-SE animals, p-GP expression is observed in endothelial cells, not in astrocytes within the hippocampus. (B) Four weeks after SE, p-GP expression is observed within the hippocampus in reactive astrocytes, not in endothelial cells. (C) In the PC, p-GP expression is observed in endothelial cells, not in astrocytes within the non-SE animals. (D) Four weeks after SE, p-GP expression is observed in endothelial cells, not in palisade astrocytes within the PC. Bar = 12.5 μ m.

more severe and prolonged than that in the hippocampus (24, 28, 29). In addition, neovascularization, astroglial loss, and the palisade reactive astroglial formation were clearly observed in the PC following SE, as compared to the hippocampus (23, 24, 26, 28). Taken together, our findings suggest that the severities of vasogenic edema and astroglial damage may affect cellular specific up-regulation of ABC family induced by SE.

Unlike other drug efflux proteins, MRP1 expression was gradually decreased and was not increased until 4 weeks after SE. Based on qRT-PCR data showing re-enhancement of its mRNA expression (18), our findings indicate that MRP1 protein translation would be slower than other drug efflux protein translation. The temporal profile of MRP1 protein expression and the cellular localization of this transporter needs to be explored.

In conclusion, the present data reveal cellular/regional specific biphasic alterations in the expression of BCRP, MRP4, and p-GP expression after SE that parallel vasogenic edema formation and recovery. Therefore, our findings suggest that the presence of vasogenic edema lesion and/or the severity of BBB disruption/reactive astrogliosis may be possible risk factors inducing pharmacoresistant epilepsy.

MATERIALS AND METHODS

Experimental animals and chemicals

This study utilized the progeny of male Sprague-Dawley (SD) rats (7 weeks old) obtained from the Experimental Animal Center, Hallym University, Chunchon, South Korea. The ani-

mals were provided with a commercial diet and water *ad libitum* under controlled temperature, humidity, and lighting conditions ($22 \pm 2^\circ\text{C}$, $55 \pm 5\%$ and a 12:12 light/dark cycle with lights). Procedures involving animals and their care were conducted in accordance with our institutional guidelines that comply with the NIH Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80-23, 1996). In addition, all possible efforts were taken to avoid animals suffering and to minimize the number of animals used at each stage of the experiment. All reagents were obtained from Sigma-Aldrich (St. Louis, MO, USA), except as noted.

SE induction

Animals were given LiCl (Sigma-Aldrich Co., St. Louis, MO, USA; 127 mg/kg i.p.) 20 hours before the pilocarpine treatment. Animals were treated with pilocarpine (25 mg/kg, i.p.) 30 minutes after scopolamine butylbromide (2 mg/kg, i.p.). Diazepam (Valium; Hoffman la Roche., Neuilly sur-Seine, France; 10 mg/kg, i.p.) was administered 2 hours after onset of SE and repeated as needed for complete remission of SE. Age-matched control (non-SE) animals received saline in place of pilocarpine and housed under the same conditions as SE animals for the same time period.

Tissue processing

At designated time points (Non-SE, 12 h, 1 day, 3 days, 4 days, 1 week and 4 weeks after SE; $n = 3$, for each time point), animals were perfused transcardially with phosphate-buffered saline (PBS) followed by 4% paraformaldehyde in 0.1 M phosphate buffer (PB; pH 7.4) under urethane anesthesia (1.5 g/kg, i.p.). The brains were removed, and postfixed in the same fixative for 4 hr. The brain tissues were cryoprotected by infiltration with 30% sucrose overnight. Thereafter, the entire hippocampus was coronally sectioned with a cryostat at 30 μm and consecutive sections were contained in six-well plates containing PBS. For stereological study, every sixth section in the series throughout the entire PC was used in some animals (29).

Double immunofluorescence study

Sections were incubated with 3% bovine serum albumin in PBS for 30 min at room temperature. Sections were then incubated in a mixture of goat anti-glucose transporter-1 (GLUT-1) IgG/rabbit anti-BCRP IgG (Acris, San Diego, CA, USA, diluted 1:100; Abbiotec, San Diego, CA, USA, diluted 1:500, respectively), mouse anti-glia fibrillary acidic protein (GFAP) IgG/rabbit anti-BCRP IgG (Chemicon, Temecula, CA, USA, diluted 1:4,000; Abbiotec, San Diego, CA, USA, diluted 1:500), goat anti-GLUT-1 IgG/rabbit anti-MRP1 IgG (Abbiotec, San Diego, CA, USA, diluted 1:100), mouse anti-GFAP IgG/rabbit anti-MRP1 IgG (Chemicon, Temecula, CA, USA, diluted 1:4,000; Abbiotec, San Diego, CA, USA, diluted 1:100), rabbit anti-GLUT-1 IgG/goat anti-MRP4 IgG (Abcam, Cambridge, UK, diluted 1:200; Origene, Rockville, MD, USA, diluted 1 : 200), mouse anti-GFAP IgG/goat anti-MRP4 IgG (Chemicon,

Temecula, CA, USA, diluted 1:4,000; Origene, Rockville, MD, USA, diluted 1:200), goat anti-GLUT-1 IgG/mouse anti-p-GP IgG (Abbiotec, San Diego, CA, USA, diluted 1:100) or mouse anti-GFAP IgG/rabbit anti-p-GP IgG (Chemicon, Temecula, CA, USA, diluted 1:4000; Abbiotec, San Diego, CA, USA, diluted 1:100) in PBS containing 0.3% triton X-100 overnight at room temperature. After washing three times for 10 minutes with PBS, sections were also incubated in a mixture of FITC- and Cy3-conjugated secondary antisera (or streptavidin; Jackson immunoresearch laboratories inc., West Grove, PA, USA; diluted 1:250) for 2 hr at room temperature. The sections were washed three times for 10 min with PBS and mounted on gelatin-coated slides. For nuclei counterstaining, we used Vectashield mounting medium with DAPI (Vector., Burlingame, CA, USA). All images were captured using an AxioImage M2 microscope and AxioVision Rel. 4.8 software (30). Fluorescence intensity was measured using a computer-assisted image analysis program (The University of Texas ImageTool program V. 3.0 and AxioVision Rel. 4.8 software).

Western blot

Tissue lysate proteins were then loaded onto a 10% polyacrylamide gel. After electrophoresis, gels were transferred to nitrocellulose transfer membranes (Schleicher and Schuell BioScience Inc., Keene, NH, USA). To reduced background staining, the filters were incubated with 5% nonfat dry milk in TBS containing 0.1% Tween 20 for 45 minutes, followed by incubation with primary antibody (see double immunofluorescence study) and subsequently with an HRP-conjugated secondary antibody (Enzo Life Science, Farmingdale, NY, USA; diluted 1:5,000). Western blotting was conducted with an ECL Western Blotting Detection Kit (Amersham) (31). Intensity measurements were represented as the mean grayscale value on a 256 gray-level scale (using ImageJ software).

Data analysis

All data obtained from the quantitative measurements were analyzed using a Kruskal-Wallis test with Mann-Whitney pairwise comparisons to determine statistical significance. In the pairwise comparisons, a Bonferroni correction was applied to take account of multiple comparisons. A *p*-value below 0.05 was considered statistically significant (32).

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