

# Reactive astrocytes increase the expression of P-gp and Mrp1 via TNF- $\alpha$ and NF- $\kappa$ B signaling

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**Abstract.** To understand multidrug resistance gene expression in reactive astrocytes, the present study involved stimulated astrocytes with tumor necrosis factor (TNF)- $\alpha$  and determined gene expression by reverse transcription-quantitative polymerase chain reaction and western blot analysis. Stimulation induced P-glycoprotein (gp) and multidrug resistance-associated protein (MRP0) 1 expression, which peaked by 24 h. Interestingly, the expression of P-gp and Mrp1 correlated with cell proliferation. Therefore, reactivation of astrocytes may increase P-gp and Mrp1 expression through TNF- $\alpha$  and nuclear factor (NF)- $\kappa$ B signaling. To the best of the authors' knowledge, the present study is the first to demonstrate that *in vitro* activated astrocytes likely induced P-gp and Mrp1 expression by increasing NF- $\kappa$ B expression. The results of the present study provide a novel insight into the mechanism of refractory epilepsy and suggest inhibiting cytokine signaling may block multidrug resistance.

## Introduction

Despite considerable advances in the pharmacotherapy of epilepsy, ~1/3 of epilepsy patients refractory to antiepileptic drugs (AEDs) do not become seizure-free (1). Therefore, seizures, which increase the risk of death from epilepsy, still pose a major health problem. Although many drugs have different mechanisms of action, a patient who is resistant to one major AED is commonly refractory to other AEDs as well. Unfortunately, the mechanisms of antiepileptic drug resistance remain unclear. However, a recently identified consequence of seizure activity that limits pharmacotherapy with antiepileptic drugs is the overexpression of multidrug transporters, such as P-glycoprotein (P-gp) and the multidrug resistance-associated protein (MRP).

Almost all insults to the brain, including prolonged seizures, result in reactive gliosis, which is characterized by severe morphological and biochemical changes of activated astrocytes (2). Astrocytes, the most abundant glial cell type in the brain, provide metabolic substrates and neurotrophic factors for neurons, control extracellular pH, potassium and glutamate levels, and participate in the formation and preservation of the blood-brain barrier (BBB). In addition, glial pathology is a universal feature of focal epilepsy (3). However, gliosis, where astrocytes can undergo morphological changes, become hypertrophied and increase in number, is not simply a response to neuronal degeneration. For instance, hypertrophy of astrocytes has been observed during the process of epileptogenesis before the development of seizures and in the absence of other pathological changes.

Tumor necrosis factor (TNF)- $\alpha$  is known to stimulate the proliferation of astrocytes; therefore, it may play a role in reactive gliosis following brain injury (4). In addition, nuclear factor (NF)- $\kappa$ B activity, which can be stimulated by TNF, has been confirmed in glial cells in neuronal plasticity and neurodegenerative disorders (5). Thus, several animal studies have demonstrated the involvement of TNF- $\alpha$  and NF- $\kappa$ B in seizure activity. One study indicates that inflammatory cytokines and related genes are involved in spontaneously recurring seizures when expressed in the hippocampus (6). NF- $\kappa$ B is a transcription factor that regulates the expression of a wide variety of genes involved in cellular events such as inflammation, immune response, proliferation, apoptosis and multidrug resistance (7-9). P-gp (ABCB1) and multidrug resistance-associated protein 1 (Mrp1 or ABCC1) are two ATP-dependent transmembrane glycoproteins involved in multidrug resistance. Initially, these glycoproteins appeared to participate in the multidrug resistance of tumor cells found in many tissues (10-12). P-gp has been characterized in the endothelial cells of brain capillaries, astrocyte foot processes associated with capillaries (13), and in primary cultures of rat astrocytes (14,15). The expression of Mrp1 has been identified in parenchyma and isolated capillaries in addition to primary rat astrocyte and neuron cultures (16-18). Interestingly, the expression of various transporters can influence the intracellular concentrations of naturally occurring compounds and pharmacological agents in astrocytes and microglia (16).

Currently, it is not clear whether the heightened expression of P-gp, also known as Mdr1, in the endothelium and parenchyma is a consequence of epilepsy, uncontrolled seizures

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and chronic AED treatment, or if the constitutive expression is present before the onset of epilepsy. Although a number of *in vitro* studies separately report that seizures induce gliosis as well as the expression of MDR or MRPs (3,19), little is known about the relationship between the two. The authors hypothesized that reactive astrocytes may increase the expression of P-gp and Mrp1 through TNF- $\alpha$  and NF- $\kappa$ B signaling based on a series of observations. First, during the continued occurrence of seizures astrocytes repeatedly undergo accretion. Moreover, hypertrophy of astrocytes during the process of epileptogenesis has been observed before the development of seizures (20). Secondly, MDR1 and MRPs are overexpressed in drug refractory epileptic tissue, and MDR1-mediated drug secretion was increased in human reactive astrocytes compared with 'normal' astrocytes (9,21). Third, the expression of MDR and MRPs is increased in primary cultures of rat astrocytes. Specifically, Gaillard *et al* (22) demonstrated increased P-gp expression in astrocytes in an *in vitro* model. Fourth, TNF- $\alpha$  is a major cytokine released during seizures that stimulates astrocyte proliferation and plays a role in reactive gliosis following seizures (23). Rizzi *et al* (24) demonstrated that both glial cell activation and cytokine expression, which included TNF- $\alpha$ , were increased in the hippocampus 4 and 24 h following kainic acid-induced status epilepticus. In the central nervous system, TNF- $\alpha$  is primarily produced by astrocytes and serves a role in cell reaction, a pathogenic mechanism of epilepsy (25). In addition, NF- $\kappa$ B, a downstream target of TNF- $\alpha$ , can regulate the expression of MDR and MRPs (26).

In the present study, the authors characterized the association between activated astrocytes and the expression of multidrug resistance genes. Cultured astrocytes were exposed to TNF- $\alpha$  for differing periods of time to achieve degrees of hyperplasia in reactive astrocytes. At each time point, the expression of MDR or MRPs was characterized by reverse transcription-quantitative polymerase chain reaction (RT-qPCR) and western blotting assays. The authors demonstrated that TNF- $\alpha$  and NF- $\kappa$ B may increase the expression of P-gp and Mrp1 in reactive astrocytes in a complex, time-dependent manner. Therefore, P-gp and Mrp1 are novel targets of a TNF- $\alpha$  and NF- $\kappa$ B-dependent signaling pathway in activated astrocytes.

## Materials and methods

**Preparation and treatment of primary rat astrocyte cultures.** Primary astrocytes were prepared from newborn Sprague-Dawley rat cerebral cortices (supplied by the University Laboratory Animal Services Centre, The Medical College of Xi'an Jiaotong University, Xi'an, China) and cultured as described previously (26). The brain was obtained from neonatal rats under sterile conditions. Following removal of the pia mater and vessels, the cortex was collected, minced mechanically on a 250- $\mu$ m nylon-mesh filter (Nippon Rikagaku Kikai Co., Ltd., Tokyo, Japan), digested in 0.25% trypsin for 30 min at 37°C, incubated with complete medium (DMEM/F12) containing 20% fetal calf serum (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) to stop digestion, and then filtered and isolated by centrifugation for 5 min at 4,000 x g at 4°C. Following differential adherence for 60 min, the cell suspension was transferred to a poly-L-lysine-coated

75 cm<sup>2</sup> culture flask. Cells at 2x10<sup>5</sup>/cm<sup>2</sup> were maintained at 37°C in a humid atmosphere with 5% CO<sub>2</sub>. The medium was changed once every 3 days. When the cells reached 70-80% confluency (~9-14 days), microglia and oligodendrocytes were removed from the astroglial bed layer following an 18 h incubation on a swing bed (960 x g) at 37°C. The adherent astrocytes were then removed with 0.25% trypsin + EDTA and replaced for experimental use. The astrocyte purity of cultures prepared in this manner was >98% as defined by staining with anti-glial fibrillary acidic protein (GFAP) antibody (Santa Cruz Biotechnology, Inc., Dallas, TX, USA).

**MTT assay for cell viability.** Following three passages, TNF- $\alpha$  was added to astrocytes at a final concentration of 2 ng/ml and incubated for 2, 24 or 48 h. The degree of proliferation and cell viability following TNF- $\alpha$  stimulation was assessed by colorimetric MTT assay. Confluent cells were cultured in 96-well plates at 5,000 cells/well and exposed to TNF- $\alpha$  for 2, 24, or 48 h. The media was discarded and the cells were washed twice with PBS before serum-free media was added back to each well. Following a 1 h incubation at 37°C, MTT 0.5 g/l was added to each well and allowed to incubate for an additional 2 h. The media was then aspirated from the plate, and 200  $\mu$ l DMSO was added to dissolve any formazan crystals. Proliferation and cell viability was determined based on the measured optical density at 490 nm using a microplate reader.

**RT-qPCR analysis.** Total cellular RNA was isolated from treated cells or control samples using TRIzol Reagent (Gibco; Thermo Fisher Scientific, Inc.). Briefly, at the designated time, cells were lysed in 1 ml TRIzol reagent. RNA was extracted from each sample by adding 0.2 ml chloroform, mixing by inversion, and shaking vigorously for 15 sec. The mixture was centrifuged, and the top aqueous phase was collected. The RNA was then precipitated overnight with 0.5 ml isopropanol at 20°C and isolated by centrifugation 8,000 x g for 20 min at 4°C the following day. The RNA pellet was washed, dried and dissolved in RNase-free water. The RNA concentration was determined by spectrophotometry at 260 nm. The purity was estimated by the ratio of A260/A280 nm, which ranged from 1.7 to 2.0.

RT-qPCR analysis for Mdr1, Mrp1, NF- $\kappa$ B and  $\beta$ -actin expression was performed according to a modified protocol (17), with specific primers for rat Mdr1 (sense primer, 5'-TCCAGCGGCAGAACAGCAAC-3'; antisense primer, 5'-GAGCAGCGTCATTGGCAAGC-3'; 231 bp), rat Mrp1 (sense primer, 5'-CAGCAGCACAGCAGCACAG-3'; antisense primer, 5'-AGGCGACGGGAGGCAAG-3'; 368 bp), rat NF- $\kappa$ B (sense primer, 5'-CACCAAAGACCCACCTCACC-3'; antisense primer, 5'-CGCACTGTCCATTGGAAGC-3'; 267 bp), and control  $\beta$ -actin (sense primer, 5'-CTATCGGCAATGAGCGGTTCC-3'; antisense primer, 5'-TGTGTTGGCATAGAGGTCTTTACG-3'; 146 bp). PCR amplification of cDNA was achieved with one cycle at 95°C for 5 min, followed by 35 cycles at 94°C for 30 sec, 56°C for 30 sec, 72°C for 30 sec used by RevertAid™ kit (Fermentas; Thermo Fisher Scientific, Inc.). The RT-qPCR products were visualized by electrophoresis in 2% agarose gel containing 0.5  $\mu$ g/ml ethidium bromide. Analysis of RT-qPCR products

was conducted by scanning densitometry with Bio-Rad ChemiDoc XRS Gel Documentation system and quantified using Bio-Rad Quantity One 1-D analysis software (Bio-Rad Laboratories, Inc.).

**Western blot analysis.** Sample preparation and western blotting was carried out as previously described (27). Protein samples were separated by SDS-PAGE, and the primary antibodies used were anti-P-gp, anti-Mrp1, anti-Gfap (Santa Cruz Biotechnology, Inc.). The membranes were blocked for 1 h at 4°C in TBS [15 mM TrisHCl, 150 mM NaCl, (pH 7.6)] containing 0.05% (v/v) Tween-20 (TBS-T) and 5% (m/v) dry skim milk powder. Following six washes (5 min each) with TBS-T, membranes were incubated with P-gp (cat. no. ABIN4904743), Mrp1 (cat. no. ABIN1584832; 1:250 in 5% milk), or  $\beta$ -actin antibody (cat. no. ABIN1742508; 1:1,000 in 5% milk) overnight at 4°C. Following a second wash, the membranes were incubated for 2 h in the presence of anti-rabbit (cat. no. A0545; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) horseradish peroxidase-conjugated secondary antibodies (1:1,000) in 5% milk at room temperature. Protein bands were visualized using an enhanced chemiluminescence (EMD Millipore, Billerica, MA, USA) western blotting analysis system (Bio-Rad Laboratories, Inc., Hercules, CA, USA) and analyzed by Image J software version 1.37 (Media Cybernetics, Inc., National Institutes of Health Bethesda, MD, USA).

**Cell immunohistochemical staining (IHC).** The treated cells were fixed with 4% paraformaldehyde and permeabilized using 0.2% Triton X-100. Then the fixed cells were incubated with P-gp, and Mrp1 primary antibody followed by streptavidin peroxidase-conjugated secondary antibody (SABC method) (28). Using previously reported immunohistochemical techniques (28), the staining was visualized by using diaminobenzidine and counterstained with hematoxylin, ten independent high-magnification fields (magnification, x400) were evaluated for each section using a laser scanning confocal microscope (TCS2 SP5; Leica Microsystems GmbH, Wetzlar, Germany).

**Statistical analysis.** Data are expressed as the mean  $\pm$  standard deviation of three separate experiments. SPSS software, 16.0 (SPSS, Inc., Chicago, IL, USA) was used for analysis. Statistical analysis was performed using an analysis of variance followed by the Bonferroni/Dunn test.  $P < 0.05$  was considered to indicate a statistically significant difference.

## Results

**TNF- $\alpha$  induces proliferation and viability in cultured astrocytes.** The effect of TNF- $\alpha$  on proliferation and cell viability is presented in Fig. 1. Astrocytes were exposed to TNF for 0, 2, 24 or 48 h, the authors demonstrated that TNF- $\alpha$  significantly increased the proliferation and cell viability at each time point.

**Reactivate astrocytes have increased P-gp and Mrp1 mRNA expression.** The level of P-gp mRNA increased over time in primary astrocytes following TNF- $\alpha$  exposure (Fig. 2A).

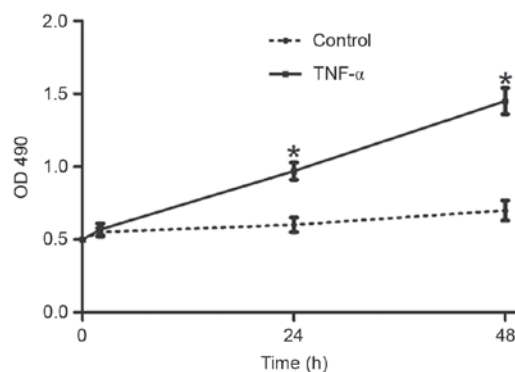


Figure 1. TNF- $\alpha$  induces proliferation and viability in cultured astrocytes (n=6). \* $P < 0.05$  vs. control. TNF- $\alpha$ , tumor necrosis factor- $\alpha$ ; OD, optical density.

While P-gp mRNA expression was detectable at each time point, the levels differed dramatically. At 2 h following TNF- $\alpha$  exposure, there was a sharp increase in P-gp expression, which peaked around 24 h before it began to decline. Compared with  $\beta$ -actin, the expression level was affected in a time-dependent manner ( $P < 0.05$ ). The timing of induced P-gp expression correlated with astrocyte proliferation, indicating that the two events may be related.

In addition, the level of MRP mRNA in primary astrocytes increased following exposure to TNF- $\alpha$  (Fig. 2B). Similar to P-gp, Mrp1 mRNA was detectable at each time point. However, 2 h following stimulation, Mrp1 expression decreased slightly. Expression then increased 24 h following TNF- $\alpha$  exposure before dropping back to a steady state level by 48 h. Again, the Mrp1 expression level changed in a time-dependent manner ( $P < 0.05$ ) that correlated with astrocyte proliferation. Therefore, Mrp1 expression and astrocyte proliferation may be related.

Consistent with a previous study, the authors noted that untreated astrocytes cultured *in vitro* expressed high amounts of Mrp1 but little P-gp (28). Surprisingly, at the mRNA level, it was reported that 2 h following TNF- $\alpha$  treatment had a greater effect on P-gp expression than Mrp1. Specifically, the expression of P-gp increased two-fold at this early time point, while there was no change in Mrp1 expression. At 24 h, P-gp expression increased seven-fold, whereas Mrp1 only increased three-fold. While the expression of both genes began to decrease following 24 h, P-gp expression was still three-fold higher at 48 h, but Mrp1 expression had reduced back to steady state levels. Therefore, changes in P-gp mRNA expression were more dramatic than Mrp1 changes in astrocytes stimulated with TNF- $\alpha$ . This may be a characteristic controlling some aspects of the multidrug resistance phenomenon.

**TNF- $\alpha$  induced P-gp and Mrp1 expression may rely on NF- $\kappa$ B expression.** The results above demonstrated that the expression of P-gp and Mrp1 is induced in response to TNF- $\alpha$  stimulation; however, other factors that regulate this response remained to be determined. Therefore, the mRNA expression of candidate regulators NF- $\kappa$ B and TNF- $\alpha$  were characterized in astrocytes following exposure to TNF- $\alpha$ . There was a significant increase in NF- $\kappa$ B expression following 2 h of TNF- $\alpha$  exposure (Fig. 3A). The expression of NF- $\kappa$ B continued to increase

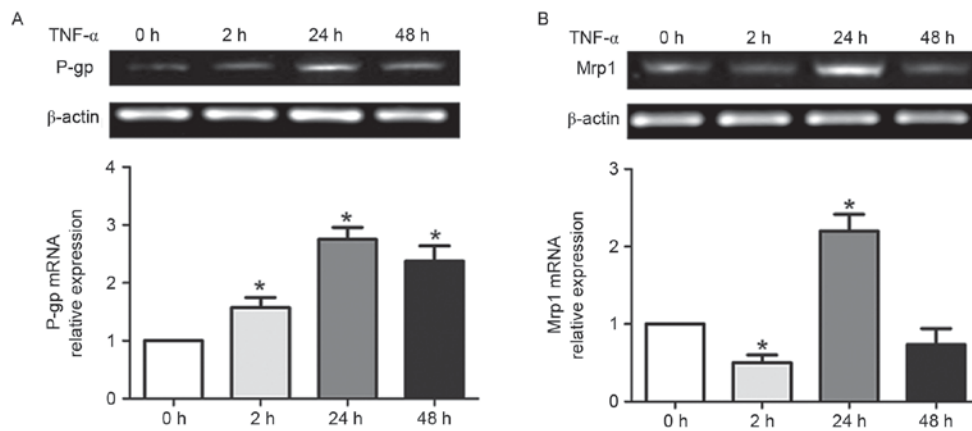


Figure 2. Reactivate astrocytes have increased P-gp and Mrp1 mRNA expression. (A) The P-gp mRNA expression after the TNF- $\alpha$  treatment. (B) The Mrp1 mRNA expression following TNF- $\alpha$  exposure (n=6). \*P<0.05 vs. 0 h. P-gp, P-glycoprotein; Mrp1, multidrug resistance-associated protein; TNF- $\alpha$ , tumor necrosis factor- $\alpha$ .

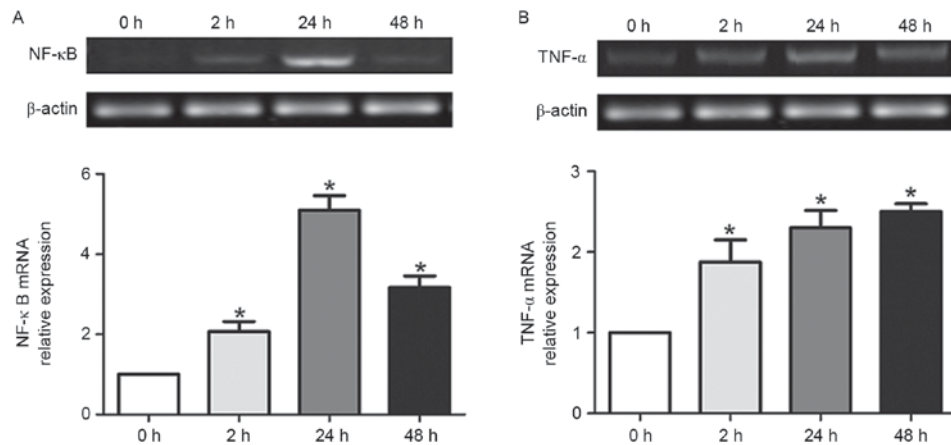


Figure 3. TNF- $\alpha$  induced P-glycoprotein and Mrp1 expression may rely on NF- $\kappa$ B expression. The (A) NF- $\kappa$ B mRNA and (B) TNF- $\alpha$  mRNA were characterized in astrocytes following exposure to TNF- $\alpha$  (n=6). \*P<0.05 vs. 0 h. TNF- $\alpha$ , tumor necrosis factor- $\alpha$ ; NF- $\kappa$ B, nuclear factor- $\kappa$ B.

through 24 h before tapering at ~48 h. These differences were statistically significant (P<0.05), which indicated that reactive astrocytes induced NF- $\kappa$ B expression in a time-dependent manner.

Interestingly, TNF- $\alpha$  exposure induces its own expression in primary astrocytes (Fig. 3B). In contrast with P-gp, Mrp1, and NF- $\kappa$ B, the expression of TNF- $\alpha$  was induced over time but did not diminish by 48 h. This may be the result of a continuous feed-forward loop in which TNF- $\alpha$  expression induces further TNF- $\alpha$  expression.

**Verification of results by western blot analysis.** To confirm these results, the protein expression of P-gp and Mrp1 was determined by western blotting. It was difficult to detect P-gp protein expression in untreated astrocytes, but expression increased following TNF- $\alpha$  exposure (Fig. 4). Mrp1 protein expression was also difficult to detect regardless of TNF- $\alpha$  exposure. Although expression was hard to detect, it was clear that the protein expression of P-gp and Mrp1 increased and peaked following 24 h of TNF- $\alpha$  exposure. Moreover, the authors performed IHC to confirm the similar results with the western blot (Fig. 5). This was in accordance with the trend of mRNA expression and astrocyte proliferation, which

indicated that reactive astrocytes may control proliferation and multidrug resistance in a similar manner.

## Discussion

The current study was undertaken to compare cell proliferation with the expression of P-gp and Mrp1 in reactive astrocytes. To the best of the authors' knowledge, the present study provides the first evidence that TNF- $\alpha$ -induced reactive astrocytes express P-gp and Mrp1, which may be dependent on induced NF- $\kappa$ B expression.

There is growing evidence that astrocytes function in many ways to support the function of the central nervous system. They appear to be intimate partners of adjacent neurons, providing them with nutrients and neurotrophic factors as well as aiding in signal transmission by ions and neurotransmitters (29). It is well known that astrocytes undergo accretion during the occurrence of continued seizures. TNF- $\alpha$  is a major cytokine released during seizures, and a regulator of reactive gliosis following seizures. Interestingly, a report by Dvorianchikova (30) demonstrated that TNF- $\alpha$  initiated the activation in astrocytes. Furthermore, Cui *et al* (31) demonstrated that TNF- $\alpha$  increased astrocyte proliferation,



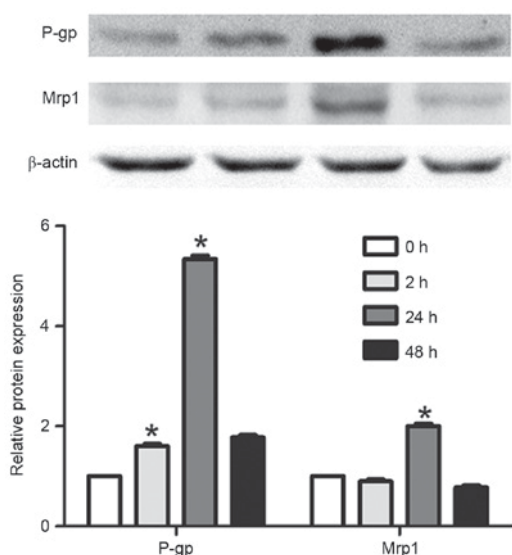


Figure 4. Western blot analysis confirmed the results. The protein expression of P-gp and Mrp1 was determined by western blot analysis after tumor necrosis factor- $\alpha$  exposure (n=6). \*P<0.05 vs. 0 h. P-gp, P-glycoprotein; Mrp1, multidrug resistance-associated protein.

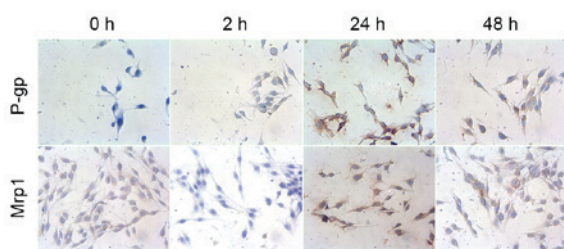


Figure 5. IHC analysis confirmed the results (magnification, x400). The protein expression of P-gp and Mrp1 was determined by IHC following tumor necrosis factor- $\alpha$  exposure (n=6). IHC, immunohistochemistry; P-gp, P-glycoprotein; Mrp1, multidrug resistance-associated protein.

which was confirmed in the present study. From the results presented in Fig. 1, it may be concluded that TNF- $\alpha$  induces reactive astrocytes to proliferate as soon as 2 h following stimulation, and cell proliferation peaks by 24 h. However, the proliferation effect subsided by 48 h post-treatment. It is possible that long exposures to TNF- $\alpha$  caused the astrocytes secrete nitric oxide and other factors related to apoptosis, which may explain why the rate of proliferation reduced. In addition, exposure to TNF- $\alpha$  increased cellular production of TNF- $\alpha$ , which may result in a positive feed-forward loop.

Brain permeability of xenobiotics, including drugs, is not only highly regulated at the brain barriers [i.e., blood-brain barrier (BBB) and blood-CSF barrier], but can also be regulated by brain parenchymal cells. For instance, astroglia can control the uptake of xenobiotics and, thus, the resulting cerebral effects (32). Therefore, the activity of membrane transporters in astroglia may be of significance in the treatment of various clinical conditions, including epilepsy. Several families of cell membrane proteins can modulate drug transport in the CNS. In particular, members of the ATP-binding cassette superfamily of transporters, such as P-gp and MRPs, can serve a major role in the effect of drugs in the brain. Although the functional expression and localization of these transporters has been

extensively examined at the brain barriers, the expression of these transporters in parenchymal cells is poorly defined. For example, the role of P-gp in astrocytes has not been clearly elucidated; although, it has been hypothesized that P-gp might limit CNS uptake of P-gp substrates due to expression in astrocyte foot processes of the brain microvasculature. In addition to astrocyte foot processes, P-gp is also expressed in astrocyte processes next to the endothelial cells of brain vessels, suggesting that the P-gp expression in astrocytes, rather than endothelial cells, maintains the BBB-function (13). The localization of Mrp1 was restricted to certain membrane areas in astrocytes, especially in the foot-processes, which suggests a possible role in physiological transport. The current data confirms a previous study demonstrating that P-gp and Mrp1 mRNAs are expressed in primary cultures of rat astrocytes (17). Furthermore, it was confirmed that the expression of Mrp1 is higher than P-gp in rat astrocytes under basal conditions (13,33).

Epileptic patient samples have increased P-gp staining in capillary endothelium and astrocytes. In addition, ten-fold increases in P-gp mRNA expression have been detected in patients with medically intractable epilepsy (9). Recent findings have demonstrated that TNF- $\alpha$  is produced both by microglia and astrocytes in adult rodent brain in various neuropathological conditions, including seizures (33). This cytokine, together with other proinflammatory molecules, appears to contribute to the mechanisms mediating excitotoxic and/or apoptotic neuronal cell death (34). However, astrocytes undergo accretion during continued seizures, and the authors observed cellular proliferation *in vitro*, rather than apoptosis. In addition, the expression of both P-gp and Mrp1 correlated with the status of astrocyte cell proliferation and activation (Figs. 2 and 3). In some pathological conditions, such as epilepsy, astrocytes are reactive and release a large amount of cytokines. Therefore, 'second-line defense' mechanisms in the perivascular glia might be induced and be responsible for poor responsiveness to medication (35,36). In addition, the change in P-gp expression in activated astrocytes was much larger than Mrp1. Under common conditions, the authors hypothesize that Mrp1 serves a major physiological transport function. Under pathological conditions, activated astrocytes proliferate and MDR expression is induced, which may be a principal factor in multidrug resistance.

P-gp and Mrp1 expression are induced in many circumstances, such as in tumor tissues, ischemia and epileptic attack. TNF- $\alpha$  and other cytokines are also produced in many of those circumstances. Many *in vivo* experiments have emphasized that TNF- $\alpha$  exposure induces P-gp and Mrp1 expressions. In addition, several animal studies have demonstrated a relationship between TNF- $\alpha$  and NF- $\kappa$ B in seizure activity (6,10). NF- $\kappa$ B is a target of TNF- $\alpha$  signaling (37), its activation induced P-gp expression in kidney proximal tubule cells (38). As presented in Fig. 3A, TNF- $\alpha$  induced a significant increase of NF- $\kappa$ B expression in astrocytes as early as 2 h following exposure, which may explain a mechanism by which downstream genes (MDR, Mrp1) are expressed. Notably, TNF- $\alpha$  can induce both anti-apoptotic and pro-apoptotic pathways. Activation of the NF- $\kappa$ B pathway results in activation of genes that mediate anti-apoptotic or protective functions. It is possible that induction of P-gp protects the cell against apoptosis. In support of

this hypothesis, a direct association between P-gp overexpression and apoptosis was established by Johnstone, Cretney and Smyth (39), who observed that a T-cell leukemia cell line transduced with a retroviral construct containing human MDR1 was more resistant to TNF- $\alpha$ -induced apoptosis than control cells (39). Therefore, these possibilities should be addressed in future experiments.

In conclusion, to investigate the association between proliferation and P-gp or Mrp1 expression in reactive astrocytes, the authors stimulated rat astrocytes with TNF- $\alpha$  for 2, 24 or 48 h to activate them. The expression of P-gp or Mrp1 was observed using RT-qPCR or western blotting, and identified that reactive astrocytes increased the expression of P-gp and Mrp1, possibly through TNF- $\alpha$  and NF- $\kappa$ B signaling. Although activated astrocytes expressed P-gp and Mrp1, the alteration in MDR expression was much larger than Mrp1. The authors suggested that, under normal conditions, Mrp1 serves a major physiological transport function in astrocytes. However, under pathological conditions, P-gp expression may be a principal agent in multidrug resistance phenomenon. These are the first findings to confirm that activated astrocyte can induce P-gp and Mrp1 expression *in vitro*. Furthermore, NF- $\kappa$ B may be the TNF- $\alpha$  target that regulates this phenomenon. The current findings offer novel insights into the cellular mechanisms that underlie refractory epilepsy and suggest that blocking cytokine signaling may reverse multidrug resistance.

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