Neuroendocrinology Letters Volume 41 No. 5 2020
ISSN: 0172-780X; ISSN-L: 0172-780X; Electronic/Online ISSN: 2354-4716
Web of Knowledge / Web of Science: Neuroendocrinol Lett
Pub Med / Medline: Neuro Endocrinol Lett

ORIGINAL ARTICLE

Influence of p-glycoprotein on brain Bcl-2 family proteins and cytokines in transient cerebral ischemia

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Submitted: 2020-09-03 Accepted: 2020-11-02 Published online: 2020-11-02

Key words: **P-glycoprotein; brain ischemia; Bcl-2; cytokines; mouse**

Neuroendocrinol Lett 2020; **41**(5):231–238 **PMID:** 33315338 NEL410520A08 • **www.nel.edu**

Abstract

OBJECTIVES: P-glycoprotein (P-gp), produced by the multidrug resistance *(mdr1a)* gene, is present in vascular endothelial cells, astrocytes, and microglia in the brain. We previously reported that P-gp aggravated cerebral infarct. Therefore, modulation of the function of P-gp is important for the treatment of brain ischemia. Here, we examined how P-gp exacerbates ischemic damage in the brain.
METHODS: Experiments were performed using mdr1a knockout (KO) mice and wild-type mice. Mice of both groups were subjected to transient focal ischemia and Bcl-2 family proteins, p-glycoprotein and cytokines were measured.
RESULTS: At 48 h after reperfusion, the expression of Bcl-2 protein in the brains of mdr1a KO mice was significantly greater compared with that of wild-type mice. The expression of brain Bax protein in mdr1a KO mice was significantly lower compared with that of wild-type mice. At 6 h after reperfusion, the expression of plasma IL-6 in mdr1a KO mice was significantly lower compared with that of wild-type mice.
CONCLUSION: These results indicate that P-gp derived from the mdr1a gene has pro-apoptotic functions mediated through Bcl family proteins and increased IL-6, which exacerbates ischemic damage in the brain. In summary, the inhibition of P-gp function is an effective strategy to protect against brain damage caused by ischemic damage.

INTRODUCTION

P-gp located in vascular endothelial cells at the blood-brain barrier (BBB) and in astrocytes and microglia in the brain parenchyma limits the entry of many xenobiotics into the brain (Lee & Bendayan 2004; Choi & Yu 2014). It was reported that the expression and activity of P-gp depend on clinical conditions including inflammation, ischemia, epileptic stroke, or brain tumor (Hermann *et al.* 2006; Miller *et al.* 2008).

Previous studies reported that the expression and activity of P-gp were increased after cerebral ischemia (Spudich *et al.* 2006; Cen *et al.* 2013); therefore, the effects of drugs (substrates of P-gp) might be insufficient to treat cerebral ischemia. We used mdr1 knockout (KO) mice to evaluate the effect of cyclosporin A, a substrate of P-gp, on cerebral ischemia in the absence of the effects of P-gp. Levels of radiolabeled cyclosporine A in the brains of mdr1a KO mice were 50-fold higher than in wild-type mice (Schinkel *et al.* 1995). Therefore, these KO mice are suitable to investigate the effect of cyclosporin A on brain tissues. In

a previous study, cyclosporin A had anti-ischemic effects and neurotoxic effects depending on the dose-response (Murozono *et al.* 2004). The mdr1a KO mouse is useful to evaluate the effects of drugs that might be substrates of P-gp and that are present at excessive intracerebral concentrations. The Mdr1a KO mouse was developed by Schinkel *et al.* (Schinkel *et al.* 1994) and is commonly used to investigate the function of P-gp derived from mdr1a. Schinkel *et al.* also reported that the mdr1a gene produced large quantities of P-gp in the BBB. The mdr1a KO mouse has also been used to examine the penetrance of drugs affected by P-gp into brain tissues during cerebral ischemia. However, it is unknown whether P-gp has any effects other than those on the intracerebral trafficking of drugs in cerebral ischemia. Therefore, we compared damage caused by cerebral ischemia in mdr1a KO mice and wild-type mice. We found that P-gp derived from mdr1a promoted damage caused by cerebral ischemia (Murozono *et al.* 2009). In general, the expressions and functions of various endogenous substances (apoptotic related factors, cytokines, neurotransmitters) were altered by cerebral ischemia and these substances influenced brain damage. Although previous studies reported the effect of P-gp on apoptosis and cytokines (Smyth *et al.* 1998; Drach *et al.* 1996; Lopes-Rodrigues *et al.* 2014; Iqbal *et al.* 2012; Kooij *et al.* 2009; Bleier *et al.* 2013), their relationship with P-gp during cerebral ischemia is unknown.

This study hypothesized that an endogenous factor regulates P-gp for cytokine (IL-1β and IL-6) and apoptosis (Bcl-2 family) production and evaluated the mechanism of P-gp brain damage during cerebral ischemia.

MATERIALS AND METHODS

Animals

Male mdr1a P-gp KO mice and age-matched wild-type control mice were purchased from Taconic Farms (Germantown, NY, USA). The experimental protocol was approved by the Tokyo Medical University Institutional Review Committee for the Use of Animal Subjects.

Transient Focal Cerebral Ischemia

According to our previously reported papers (Murozono *et al.* 2004; Murozono *et al.* 2009; Sakai *et al.* 2001), anesthesia was maintained with 1.5%–2.0% isoflurane in 67% N_2O and 34% O_2 employing an inhalation mask during the surgical procedure. A 6-0 siliconized filament (PDS®II, Ethicon, NJ, USA) was inserted into an incision in the right external carotid artery (ECA). The filament was advanced through the origin of the anterior cerebral artery to occlude the right middle cerebral artery. After a 30-min occlusion period, reperfusion was accomplished by withdrawing the intraluminal filament from the right ECA. Pre-ischemia mice underwent the same surgical procedure but without middle cerebral artery obstruction.

Assessment of Cerebral Blood Flow (Murozono et al. 2009)

To monitor regional cerebral blood flow (rCBF) continuously, a laser-Doppler flowmetry probe (EG fine probe, Omegawave, Tokyo, Japan) was fixed to the intact skull. Steady-state baseline values were recorded before MCA occlusion, and rCBF during and after occlusion was expressed as a percentage of the baseline values.

Neurological Assessment

The functional effects of ischemia were measured at 2 h after ischemia by a blinded observer. We estimated the severity of the neurological deficit using a modified 4-point scale (Murozono *et al.* 2009).

Western blot analysis

a) Bcl-2 and Bax (San-Emeterio & Hurle 2006)

Brains were obtained from mdr1a KO mice and wild-type mice treated pre-ischemia at 4 and 48 h after reperfusion (n=5 per group). The right ischemic hemisphere was dissected on ice and sonicated twice for 10 seconds each time in 10 mM Tris-HCl (pH 6.8) with 1 mM EDTA buffer, 2% sodium dodecyl sulfate (SDS), and protease inhibitors (phenylmethylsulfonyl fluoride 1.3 mM; leupeptin 10 μg/ml; pepstatin A 10 μg/ml; aprotinin 5 μg/ml; and antipain 10 μg/ml). Lysates were boiled for 10 min, sonicated, and then centrifuged at 5000 ×g for 5 min. The supernatant fluid was the total cell lysate. The protein concentration in the solution was measured by Bio-Rad Protein Assay (Bio-Rad Laboratories, CA, USA) using bovine serum albumin (Sigma-Aldrich, MO, USA) as a standard. Identical amounts (20 μg) of whole-cell protein from each sample were loaded on 12.5% SDS-polyacrylamide gel (SDS-PAGE), electrophoresed, and transferred to a polyvinylidene difluoride (PVDF) membrane (Bio-Rad, Hercules, CA, USA). The following antibodies were used: polyclonal anti-Bcl-2 (1:1000; Santa Cruz Biotechnology, TX, USA), polyclonal anti-Bax (1:1000; Abcam Cambridge, UK) and β-actin antibody (1:500; Santa Cruz Biotechnology, TX, USA). The blots were incubated with the appropriate peroxidase-labeled secondary antibody for 1 h at room temperature. Immunoreactivity was visualized with Lumi-Light Plus Western Blotting Substrate (Roche, Basel, Switzerland) and the blots were quantified with a chemiluminescence imaging system (Light capture, ATTO, Tokyo, Japan).

b) p-glycoprotein (Zhao et al. 2002)

Brains were obtained from mdr1a KO mice and wild-type mice treated pre-ischemia at 3 and 24 h after reperfusion (n=5 per group). The right ischemic hemisphere was dissected on ice and suspended in 1 ml of 10 mM Tris-HCl buffer (pH 8.0) containing protease inhibitor, phosphatase inhibitor (0.3 ml/50 ml), aprotinin 1.5 μg/ml, and 1 mM phenylmethylsulfonyl fluoride (PMSF). After the suspension was frozen at −80°C, it was sonicated and centrifuged at 2000 ×g for 15 min at 4°C.

The harvested supernatant was centrifuged again at 80000 ×g for 60 min at 4°C. The pellet was dissolved in homogenization buffer. The protein concentration in the solution was measured by Bio-Rad Protein Assay (Bio-Rad Laboratories CA, USA) using bovine serum albumin (Sigma-Aldrich, MO, USA) as a standard. Protein (40 μg) was separated by electrophoresis on a 5% SDS-polyacrylamide gel and transferred to a PVDF membrane. The membrane was blocked in PBS containing 0.1% Tween 20 and 5% BSA, and detected by C-19 polyclonal antibody (1:1000; Santa Cruz Biotechnology, TX, USA). Western blot analysis was performed using a β-actin antibody (1:400) as a loading control. As described above for the measurement of Bcl-2 and Bax by western blot, immunoreactivity was visualized and the blots were quantified.

Measurement of serum cytokine levels

Blood samples were obtained from the inferior vena cava of mice under deep general anesthesia at 3 h after ischemia for IL-1β and at 6 h for IL-6, and then serum was separated. IL-1β and IL-6 levels in the serum were measured using an ELISA Kit (Quantikine, R&D Systems, MN, USA), according to the manufacturer's instructions.

Statistical analysis

Values are expressed as the means ± SD in the text and figures. The protein levels of Bcl-2 and Bax on immunoblots were calculated as percentages of the pre-ischemia group of KO or wild-type mice. The density of P-gp bands was normalized to the pre-ischemia group of wild-type mice. Two-way ANOVA followed by post-hoc Tukey's test was used to determine the statistical significance of differences of P-gp, Bcl-2, and Bax within each group and the Mann-Whitney *U*-test was used to compare the wild-type and KO groups for each measurement item. $p<0.05$ was considered statistically significant. Statistical tests were performed using Graph Pad Prism version 7.02 for Windows (Graph Pad Software).

RESULTS

rCBF was decreased to 15%–30% of the baseline immediately after MCA occlusion and was sustained during 30 min of ischemia in KO (24.3±11.3%) and wild-type mice (21.4±6.2%). After reperfusion, the rCBF increased to 60%–80% of the baseline (KO: 69.1±19.3%, wild-type: 72±18.7%). During and after ischemia, there were no significant differences between the groups. Approximately 2 h after ischemia, the neurological scores of both groups were not significantly different (KO: 1.89±0.52, wild-type: 1.81±0.58).

Bcl-2 and Bax levels in mouse brain (Fig. 1)

Bcl-2 levels in the KO group were not significantly changed over time (pre: 100±15.7%, 4 h: 122.3± 10.3%, 48 h: 114±8%), whereas Bcl-2 levels in the wild-type group tended to be reduced over time (pre: 100±19.9%,

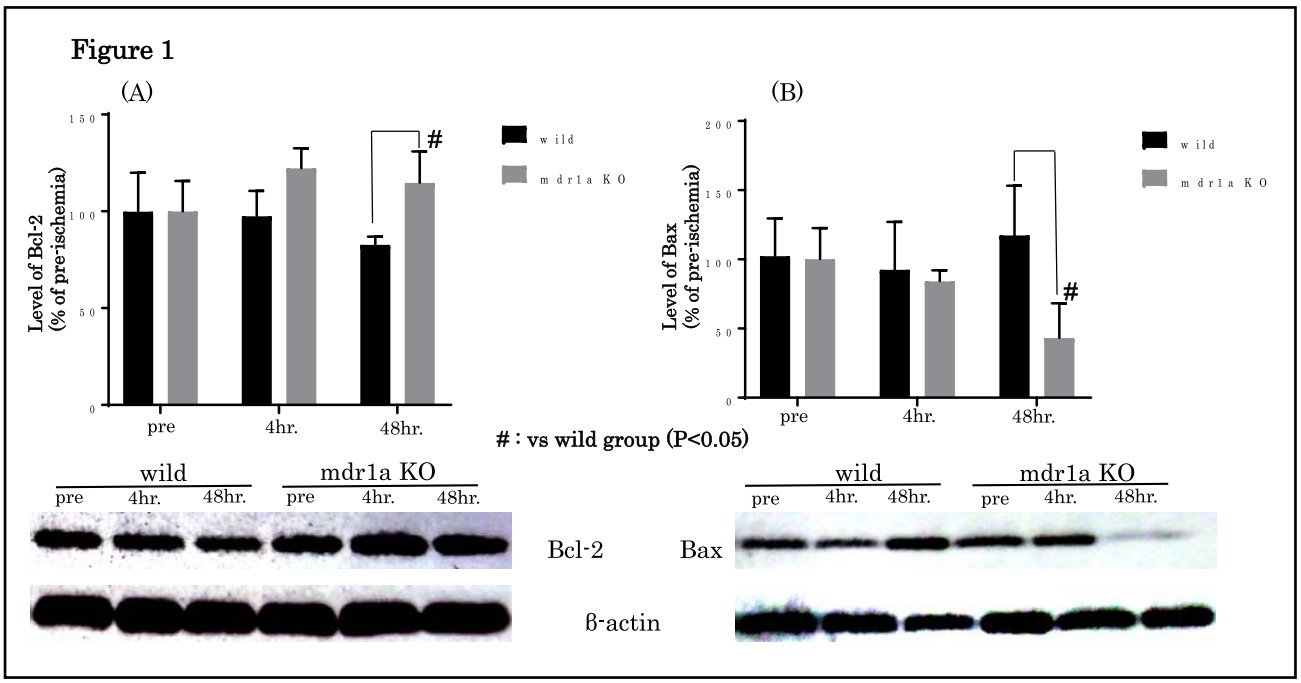

Fig. 1. Changes in brain Bcl-2 family proteins.
A, Relative changes in Bcl-2 protein expression. At 48 h after ischemia, the Bcl-2 levels of mdr1a KO mice were significantly higher compared with those of wild-type mice. B, Relative changes in Bax protein expression. At 48 h after ischemia, the Bax levels of mdr1a KO mice were significantly lower compared with those of mdr1a KO mice at pre-ischemia. Data are expressed as the mean ± SD of the % of pre-ischemic animals. Under the graphs, representative western blots show the immunoreactivity of Bcl-2 and Bax proteins in brains of mdr1a KO mice and wild-type mice at pre-ischemia and 4 and 48 h after ischemia.

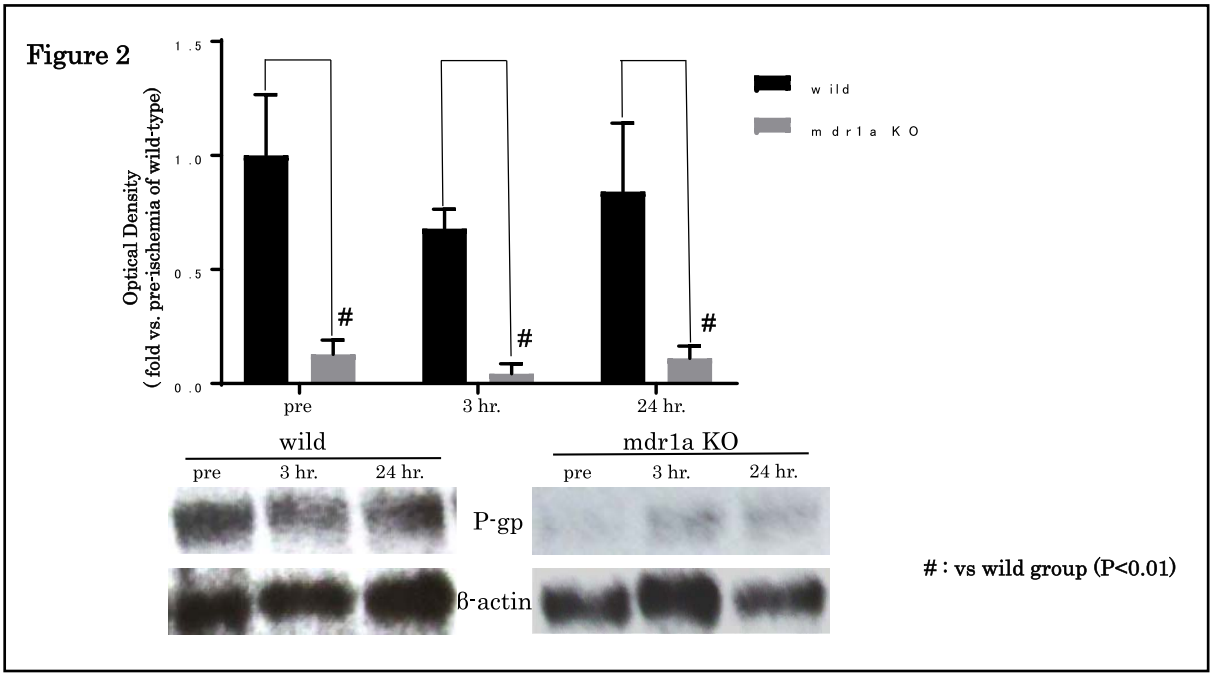

Fig. 2. Relative changes in P-gp expression.
The P-gp levels in mdr1a KO mice were significantly lower compared with those of wild-type mice at each time point. In wild-type mice, no significant change in P-gp levels was found. Data are expressed as the mean ± SD of the ratio of pre-ischemia in wild-type mice. Under the graphs, representative western blots show the immunoreactivity of P-gp proteins in brains of mdr1a KO mice and wild-type mice at pre-ischemia and at 3 and 24 h after ischemia.

4 h: 97.4±12.9%, 48 h: 82±4.1%) (Figure 1(A)). At 48 h after ischemia, Bcl-2 levels in KO mice were significantly higher than those of wild-type mice (p<0.05). There was a statistically significant difference in the Bcl-2 level in brain cells after cerebral ischemia between the wild-type group and the KO group (p<0.01).

Bax levels in the KO group were progressively reduced over time (pre: 100.2±22.4%, 4 h: 84.3±7.9%, 48 h: 43.3±24.9%) (Figure 1(B)). At 48 h after ischemia, Bax levels in KO mice were significantly lower compared with those of KO mice at pre-ischemia (P<0.05). Bax levels in the wild-type group showed no significant changes over time (pre: 102.4±27.2%, 4 h: 92.6±34.4%, 48 h: 117.5±35.9%) (Figure 1(B)). At 48 h after ischemia, Bax levels in KO mice were significantly lower than those of wild-type mice (p<0.05). There was a statistically significant difference in Bax levels in brain cells after cerebral ischemia between the wild-type group and the KO group (p<0.01).

P-gp levels in the brain (Fig. 2)

As shown in Figure 2, the P-gp levels in the KO group were unchanged with very low optical density (pre: 0.13±0.062, 3 h: 0.04±0.043, 24 h: 0.11±0.054). In the wild-type group, the P-gp levels at 3 and 24 h after ischemia tended to be decreased although this was not statistically significant (pre: 1.00±0.267, 3 h: 0.679±0.86, 24 h: 0.842±0.299). The P-gp level of wild-type mice was significantly higher compared with that of KO mice at each time point (p<0.05). There was a statistically significant difference in the P-gp level in brain cells after cerebral ischemia between the wild-type group and the KO group (p<0.001).

Serum IL-1β and IL-6 levels in Mdr1a KO mice and WT mice (Fig. 3)

As shown in Figure 3(A), there was no difference in IL-1β levels between both groups (KO: 53.1±36.9 pg/ml, wild-type: 43.5±31.2 pg/ml). As shown in Figure 3(B), IL-6 in KO mice (8.7±4.5 pg/ml) was significantly lower compared with that of wild-type mice (27.7±4.6 pg/ml) (p<0.01).

DISCUSSION

Many therapeutic drugs have been developed for cerebral ischemia; however, few of these are effective in the clinic. The major reason for the failure of drug therapy is the weak permeability of the drugs across the BBB. P-gp is highly expressed at the BBB and functions as a drug efflux pump, suggesting it might be an influential factor for the treatment of central nervous system diseases (Cordon-Cardo *et al.* 1990; Gottesman *et al.* 2002; Choi & Yu 2014). P-gp has many substrates, which are used as therapeutic agents via multidrug transporters expressed in the brain (Loscher & Potschka 2005). Although the tight junctions of the BBB are disrupted after ischemia, the expression and activity of P-gp were not decreased and therapeutic drugs that are P-gp substrates were not effective after ischemia (Hermann *et al.* 2006; Spudich

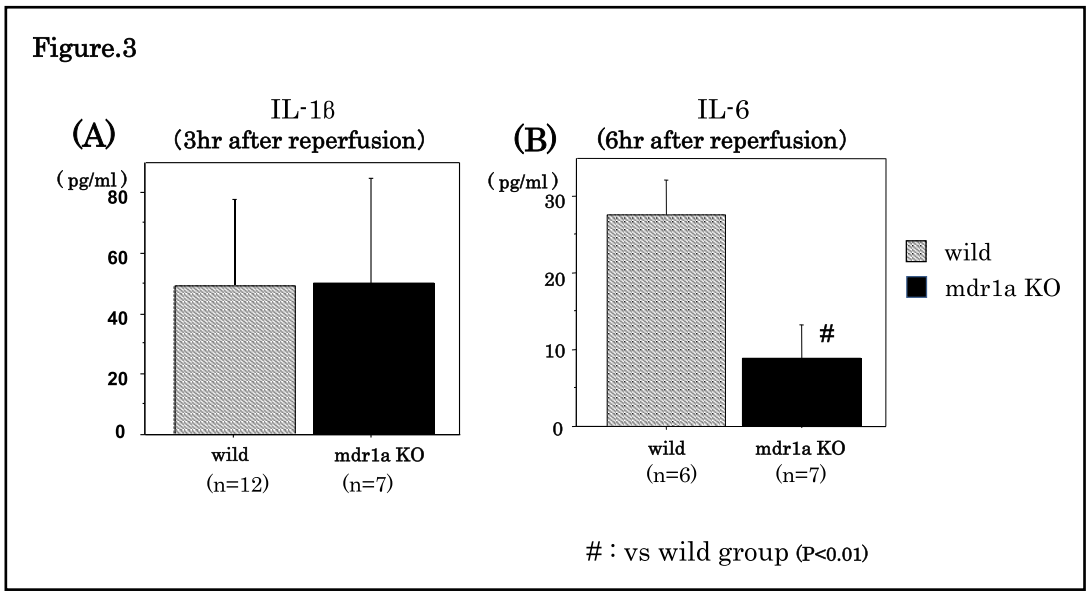

Fig. 3. IL-1β and IL-6 levels in serum after ischemia.
A, There was no significant difference in IL-1β levels between mdr1a KO and wild-type mice. B, The IL-6 levels of mdr1a KO mice were significantly lower compared with those of wild-type mice. Data are the mean ± SD.

et al. 2006; Cen *et al.* 2013). Therefore, it is important to investigate how P-gp affects brain ischemia to develop strategies to improve drug therapy. Focusing on the above points, we previously tried to increase the therapeutic effect against ischemia by suppressing the action of P-gp (Murozono *et al.* 2017).

In the present study, mdr1a KO mice and wild-type mice were subjected to transient focal cerebral ischemia and Bcl-2 family proteins and cytokines were measured to examine how P-gp influences ischemic damage in the brain. At 48 h after 30 min-ischemia, the expression of Bcl-2 protein in the brains of mdr1a KO mice was significantly higher and the expression of Bax protein was significantly lower than in wild-type mice. The expression of P-gp protein in the brains of wild-type mice was greater than in mdr1a KO mice at each time point. In wild-type mice, the expressions of P-gp protein at 3 and 48 h after ischemia were unchanged compared with its expression pre-ischemia. At 6 h after 30 min-ischemia, the serum level of IL-6 in mdr1a KO mice was significantly lower compared with that of wild-type mice. As described above, P-gp derived from mdr1a influenced the expressions of Bcl-2 family members and IL-6 in a mouse model of transient local cerebral ischemia.

Previous studies investigated the expression and function of P-gp related to brain ischemia or hypoxia (Spudich *et al.* 2006; Lazarowski *et al.* 2007; Dazert *et al.* 2006; Samoto *et al.* 1994; Cen *et al.* 2013). P-gp disturbed neuroprotective treatment after ischemia because P-gp is upregulated within a few hours after ischemia (Spudich *et al.* 2006; Lazarowski *et al.* 2007; Cen *et al.* 2013). However, other studies reported that P-gp expression was not significantly increased after brain ischemia (Dazert *et al.* 2006; Samoto *et al.* 1994). These variable results may be attributed to differences in the ischemic models used, the methods of measurement, and the brain regions assayed. The current study did not show a clear increase in P-gp expression after ischemia in wild-type mice, although P-gp is thought to be present in wild-type mice and might therefore influence brain tissues just after reperfusion as opposed to mdr1a mice, in which P-gp is not present and brain tissues are not influenced. Other reports suggested that during brain ischemia, the reduced expression and activity of P-gp led to the penetrance and accumulation of neuroprotective agents in brain tissues (Spudich *et al.* 2006; Cen *et al.* 2013). We previously reported that P-gp aggravated cerebral ischemic damage (Murozono *et al.* 2009). We measured each item under conditions identical to the previous experimental model (30-min cerebral ischemia induced by monofilament in mdr1 KO mice). We estimated that the cerebral ischemic damage was higher in mdr1a KO mice than in wild-type mice in this study, which suggests that inhibiting the functions of P-gp derived from mdr1a enhances cerebroprotection through the regulation of Bcl family proteins and IL-6 expression.

During brain ischemia, apoptosis mediated by Bcl-2 family members proceeds via mitochondria in cells. Bcl-2, Bcl-xL, Bcl-w, and Mcl-1 inhibit apoptosis, whereas Bax, Bik, Bad, and Bcl-xs, activate apoptosis (Nagata 1997; Nakka *et al.* 2008). The various Bcl-2 family members can dimerize with each other, with one monomer antagonizing or enhancing the function of the other. In this way, the ratio of inhibitors to activators in a cell may determine the propensity of the cell to undergo apoptosis (Yang & Korsmeyer 1996; Ferrer & Planas 2003). For example, if either *bcl-x* or *bcl-2* is disrupted in mice, the animals die as embryos or postnatally, respectively, as the result of excessive programmed

cell death in particular organs (Veis *et al.* 1993). Conversely, if Bax is disrupted, normal programmed cell death fails to occur (Knudson *et al.* 1995). The upregulation of Bax was reported after ischemia in association with reduced Bcl-2 in the infarct core (Matsushita *et al.* 1998; Krajewski *et al.* 1995). In contrast, the expression of Bcl-2 was increased in neurons of the penumbra (Isenmann *et al.* 1998).

In this study, the presence of mdr1a-derived P-gp indicated that Bcl-2 was decreased and Bax was increased after ischemia suggesting pro-apoptotic effects. This indicated that mdr1a-derived P-gp aggravated ischemic damage (Murozono *et al.* 2009). Studies also reported that P-gp has anti-apoptosis (Gollapud & Gupta 2001; Johnstone *et al.* 1999) and pro-apoptosis effects (Park *et al.* 2006). These reports were mainly *in vitro* studies. Therefore, these conditions may be considerably different from *in vivo* studies, such as our model. In the present study, mdr1a-derived P-gp influenced Bcl-2 family members. However, it is unknown what type of signal from P-gp acts on Bcl-2 family members during cerebral ischemia. Recently, studies of interactions between P-gp and micro-RNAs reported that P-gp may regulate the expression of miRNAs that regulate apoptosis and drug resistance (Lopes-Rodrigues *et al.* 2014). Furthermore, miR-16 downregulated Bcl-2 and promoted apoptosis (Han & Chen 2015; Rui *et al.* 2018) and was increased in ischemia (Spinetti *et al.* 2013; Tian *et al.* 2016). Another study reported that P-gp promoted apoptosis through miR-16 and Bcl-2 (Tsang *et al.* 2011). Taken together, it may be necessary to investigate the relationship between micro-RNAs and P-gp in cerebral ischemia.

Brain ischemia and late reperfusion induce an inflammatory response that is initiated in the microcirculation, which leads to neuronal destruction (Vila *et al.* 2000; Castellanos *et al.* 2002). Neurons, astrocytes, microglia, and especially endothelial cells, are activated in response to ischemia and release cytokines (DeGraba 1998; Hallenbeck 1996). IL-1β and tumor necrosis factor-α (TNF α) are key cytokines in this very early inflammatory response (Hallenbeck 1996). IL-1β and TNF-α induce a prolonged secondary inflammatory response, which is maintained by IL-6 and IL-8. These cytokines play an important role in the development of acute phase reactants, such as fever, CRP, and fibrinogen, and in the release of cell adhesion molecules, which contribute to leukocyte aggregation and adhesion to the vascular wall (Rodriguez-Yanez & Castillo 2008).

A previous study reported the over-expression of IL-1β in the brain very early (within 1 h) after the insult, which persisted during the development of the infarction (Zhang *et al.* 1998). Peripheral blood levels of IL-6 were higher in patients with stroke and were detectable within a few hours (Tarkowski *et al.* 1995). Clark *et al.* reported that serum levels of IL-6 (ELISA) peaked at 6 h after ischemia in a mouse focal reversible stroke model (Clark *et al.* 1999). Based on these reports, we measured IL-1β levels at 3 h and IL-6 levels at 6 h after reperfusion. For IL-1β, there was no significant difference between mdr1a KO mice and wild-type mice at 3 h after ischemia; however, the serum level of IL-6 in wild-type mice, which have mdr1a-derived P-gp, was significantly higher compared with that of mdr1a KO mice at 6 h after ischemia. In our previous study, the presence of mdr1a-derived P-gp increased ischemic damage (Murozono *et al.* 2009), indicating that it increases ischemic damage and serum levels of IL-6 concomitantly after ischemia. This condition is consistent with reports showing a significant correlation between plasma IL-6 and infarct size (Fassbender *et al.* 1994; Hotter *et al.* 2019). Studies also reported that P-gp contributed to the release of cytokines (Raghu *et al.* 1996; Kooij *et al.* 2009). Bleier *et al.* reported that p-gp was involved in the release of IL-6 (Bleier *et al.* 2013). The current study suggests that P-gp increases IL-6 during ischemia and contributes to the expansion of cerebral damage. However, the source of IL-6 induced by P-gp is still unknown. P-gp is mainly localized at the luminal membrane of capillary endothelial cells (Cordon-Cardo *et al.* 1989). Therefore, P-gp may induce the release of IL-6 from capillary endothelium cells during cerebral ischemia.

Here, we investigated the effect of P-gp in cerebral ischemia using mdr1a KO mice. The results showed that intracerebral P-gp promoted brain damage through apoptosis or cytokines during cerebral ischemia. The mdr1a KO mouse model is useful for establishing cerebroprotection strategies that control P-gp. Future studies should investigate the effect of P-gp inhibitors on cerebral ischemia by using mdr1a KO and normal mice. The combination of P-gp inhibitor and neuroprotective agents, which are influenced by P-gp, may lead to breakthrough neuroprotective effects that reduce ischemic damage.

ACKNOWLEDGEMENTS

We thank J. Ludovic Croxford, PhD, from Edanz Group (https://en-author-services.edanzgroup.com/ac) for editing a draft of this manuscript. The authors also thank Yasuo Watanabe for his assistance with reviewing the manuscript.

FUNDING

This work was supported by a Grant-in-Aid for Scientific Research (C) from the Japanese Ministry of Education, Culture, Sports, Science and Technology (No. 20591818: M.M.).

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