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Short Communication

Glutathione depletion upregulates P-glycoprotein expression at the blood–brain barrier in rats

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Abstract

Objectives Glutathione (GSH) depletion has been implicated in the pathogenesis of neurological diseases. During GSH depletion, cells of the blood–brain barrier are subjected to chronic oxidative stress. Using an in-vivo system, we have investigated whether glutathione depletion changed expression of P-glycoprotein at the blood–brain barrier in rats.

Methods Diethyl maleate was intraperitoneally injected to induce GSH depletion in rats. P-glycoprotein expression at the blood–brain barrier was examined by Western blotting and RT-PCR, and its function was assessed by measuring the brain-to-plasma concentration ratios (Kp values) of rhodamine 123 (Rh123). Evans Blue dye was used as a blood–brain barrier indicator for examining the extravasation from the blood to the brain.

Key findings Four hours after treatment of rats with diethyl maleate, the brain GSH content significantly reduced. The mdr1a mRNA expression at the blood–brain barrier was upregulated, whereas no significant change in mdr1b mRNA expression was found. The P-glycoprotein level was significantly increased compared with control rats. At the same time, the Kp values of Rh123 suggested that function of P-glycoprotein was significantly enhanced at the blood–brain barrier in rats with GSH depletion induced by diethyl maleate. No significant difference of the Evans Blue dye concentration in the brain cortex was found between GSH depletion rats and control rats. Treatment of rats with *N*-acetylcysteine decreased P-glycoprotein upregulation induced by diethyl maleate.

Conclusions The oxidative stress induced by GSH depletion played a positive role in the regulation of function and expression of P-glycoprotein at the blood–brain barrier in rats.
Keywords blood–brain barrier; glutathione; oxidative stress; P-glycoprotein

Introduction

P-glycoprotein (P-gp), the product of the multidrug resistance (mdr) gene, is highly expressed on the luminal surface of capillary endothelial cells.[1] Due to its transmembrane localization, P-gp functions as an efflux transporter that can limit cellular uptake of drugs from the blood circulation into the brain. To date, it has been shown that some drugs are substrates of P-gp, for example, morphine, phenytoin, flesinoxan, anticancer drugs and anti-HIV drugs.[2–5] It has been suggested that P-gp plays an important role in the transport of lipid (such as cholesterol and phosphatide), endogenous opium peptide and naturally-occurring glucocorticoid cortisol, and in regulation of lipid metabolism, the function of the algesthesis pathway and the behavioural response of glucocorticoids in the hippocampus.[6,7] In addition, abnormal expression of P-gp at the blood–brain barrier is related to some brain diseases, such as Alzheimer's disease, Parkinson's disease and epilepsy.[8–12]

More studies have shown that the presence of surrounding brain parenchyma may be important for P-gp expression, and removal of endothelial cells from their brain environment results in decreased P-gp expression.[13,14] Intracellular reactive oxygen species (ROS) have been implicated in the regulation of P-gp. Conditions that generate ROS have been shown to increase P-gp expression in cells derived from liver and kidney.[15–18] Studies with primary cultured rat brain endothelial cells showed that noncytotoxic concentrations of H_2O_2 could bring about increases in P-gp expression

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mediated, at least in part, by increased transcription with concomitant increases in functionality.[19] We previously demonstrated, by RT-PCR analysis in rat brain microvessel endothelial cells, that glutamate enhanced rat mdr1a and mdr1b mRNA levels, and that this phenomenon was inhibited by a noncompetitive *N*-methyl-D-aspartate (NMDA) receptor antagonist, MK801, and ROS scavenger, *N*-acetylcysteine.[20] We also found that P-gp expression was upregulated by glutathione (GSH) depletion-induced oxidative stress in the rat brain microvessel endothelial cells.[21]

GSH is an ubiquitous thiol-containing tripeptide that may play a crucial role in antioxidant defence mechanisms. It modulates the cell response to redox changes, detoxifies the metabolites of drugs, regulates gene expression and apoptosis, and is involved in the transmembrane transport of organic solutes. Owing to its reactivity and high intracellular concentrations, GSH has been implicated in the resistance to several chemotherapeutic agents.[22] A recent study showed that GSH and thiolated chitosan inhibited multidrug resistance P-gp activity in excised small intestine.[23] A knowledge of P-gp expression is therefore of key interest in GSH depletion, especially at the blood–brain barrier. In this study, we have investigated *in vivo* whether GSH depletion induced by a GSH exhaustion agent, diethyl maleate (DEM), could also regulate the expression and function of P-gp at the blood–brain barrier in rats.

Materials and Methods

Materials

DEM was from Changzhou Pharmaceutical Factory (Changzhou, Jiangsu, China); P-gp antibody was from Wuhan Boster Co. (Wuhan, China); *N*-acetylcysteine, Evans Blue dye and rhodamine 123 (Rh123) were obtained from Sigma Chemical Co. (St Louis, MO, USA); GSH assay kit was from Jiancheng Biotech, Nanjing, China; RT-PCR agents were purchased from Amersco Inc, (Dallas, TX, USA) and Bio Basic Inc. (Ontario, Canada). All other chemicals were of analytical grade and commercially available.

Animal preparation

All animal experiments were conducted according to the guidelines of local animal use and care committees and executed according to National Animal Law, and according to National Institutes of Health guidance for the care and use of laboratory animals.

Male Sprague-Dawley rats (160–180 g) were supplied by Jiangsui Center of Experimental Animals. The rats were housed under controlled environmental conditions (temperature, 23 ± 1°C; humidity, 55 ± 5%) with a commercial food diet and water freely available. The animal model was made as described previously.[24,25] Briefly, the rats were injected with DEM (912 mg/kg, dissolved in peanut oil, i.p.), and the control rats were injected intraperitoneally with an equal volume of peanut oil. Rats were killed 4 h after DEM treatment. *N*-acetylcysteine was administered (100 mg/kg, i.p.) 1 h after DEM treatment.

Assay for brain glutathione level

Four hours after the injection of DEM the rats were killed. The brains were removed quickly, cleaned using cold salt solution, and homogenized with salt solution (w/v, 1 : 9) in a glass homogenizer. After centrifuging the homogenized brain tissue at 2500 rev/min for 15 min, the GSH content of the supernatant was measured by a spectrometer (λ = 412 nm), and corrected by the protein content per gram.

Western blotting

The rat brain capillaries were prepared using a procedure described previously.[21,26] The purity of brain capillaries was >95% as determined by the enzyme markers γ-glutamyl trans-peptidase and alkaline phosphatase. Microvessels from the cortex were homogenized in cold lysis buffer containing 150 mM NaCl, 50 mM Tris pH 7.4, 0.1% sodium dodecyl sulfate (SDS), 1% Triton X-100, and protease inhibitors (Shanghai Sangon Corp, Shanghai, China). The samples (30 μg protein/sample) were subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE, 12%) followed by electrophoretic transfer to nitrocellulose membranes. Nonspecific binding sites were blocked by incubating the nitrocellulose membranes for 1 h in phosphate-buffered saline (PBS) containing 5% nonfat dried milk and 0.2% Tween 20 (blocking buffer). The membranes were incubated overnight at 4°C with 1 μg/ml P-gp antibody diluted in the blocking buffer. After removal of the primary antibody, the membranes were washed with blocking buffer and incubated for 1 h at room temperature with a secondary, horseradish peroxidase-coupled, IgG antibody (dilution 1 : 5000). The membranes were washed in PBS, and P-gp bands were visualized using an enhanced chemiluminescence (ECL) Western blotting analysis system (Amersham International GE Healthcare, Buckinghamshire, UK).

RT-PCR analysis

Total cellular RNA was isolated from brain cortex of DEM-treated rats and control group rats using Trizol reagent. All samples were checked for purity and relative content of RNA by UV spectrometry at 260 and 280 nm, and volumes were adjusted accordingly before proceeding to RT-PCR analysis. cDNA prepared from 20 μg total RNA of brain tissue by reverse transcription was used for PCR amplification with specific primers for rat mdr1a (sense primer: 5′-GGCCACATGATCAAGACG-3′; antisense primer: 5′-AACAAGTTGCTGTTCTGCC-3′, 243 bp, nucleotides 447–690 in S66618, GeneBank), mdr1b (sense primer: 5′-AGTGACACTGGTGCCTCT-3′; antisense primer: 5′-GTTACAATTCCGTTGTTTGG-3′, 320 bp, nucleotides 1965–2285 in NM 012623, GeneBank) and control β-actin (sense primer: 5′-CCAGAGCAAGAGAGGCATCC-3′; antisense: 5′-GCTGGGGTGTTGAAGATCTC-3′, 682 bp, nucleotides 1502–2184 in J00691, GeneBank). PCR amplification of cDNA was run at 95°C for 30 s, 55°C for 30 s and 72°C for 30 s, through a total of 40 cycles. The PCR products were separated by electrophoresis through 2% (w/v) agarose, visualized under UV light in the presence of ethidium bromide, photographed, and analysed using a UV

gel documentation system. The PCR products were normalized against β-actin mRNA.

Measurement of rhodamine 123

Four hours after injection of DEM, Rh123 (0.2 mg/kg) was injected into the tail vein, and blood and brain samples taken 45 min later. Brain cortex weighing 0.3 g was homogenized in purified water. Brain and whole blood samples were centrifuged for 5 min at 12 000 rev/min. The resulting supernatants of the samples were dissolved into purified water to make the volume sufficient for assay.[27] The fluorescence of Rh123 in plasma and brain cortex was determined by fluorescence spectrophotometry (RF-5301PC, Shimadzu, Kyoto, Japan) (excitation wavelength 485 nm; emission wavelength 535 nm).

Leakage of Evans Blue from brain vessels

Four hours after DEM treatment Evans Blue (80 mg/kg) was injected into the tail vein of all rat groups. Brain samples were taken 60 min later. Brain cortex weighing 0.25 g was homogenized in 50% trichloroacetic acid and centrifuged for 20 min at 1000 rev/min. The resulting supernatants of the samples were diluted with ethanol, and the fluorescence of Evans Blue in brain cortex was detected by fluorescence spectrophotometry (excitation wavelength 620 nm; emission wavelength 680 nm). The concentrations of Evans Blue were calculated by standard curve. The results were expressed in microgram Evans Blue per gram cortex.

Statistical analysis

Data were represented as mean ± SD and analysed by multifactorial analysis of variance, Student's *t*-test and Dunnett's test.

Results

Brain glutathione level in rats treated with diethyl maleate

Brain GSH level was significantly decreased by 34% 4 h after the intraperitoneal injection of DEM to rats, compared with control rats injected with vehicle alone (Figure 1).

Effect of glutathione depletion on permeability of the blood–brain barrier in rats

The assay for leakage of Evans Blue from brain vessels showed that the concentration of the dye in the brain cortex was 7.44 ± 1.99 μg/g in the GSH depletion group and 7.20 ± 2.85 μg/g in the control group (mean ± SD, $n = 6$). There was no significant difference between the two groups, which suggested the integrity of the blood–brain barrier was not changed after DEM treatment, used to deplete GSH in rats.

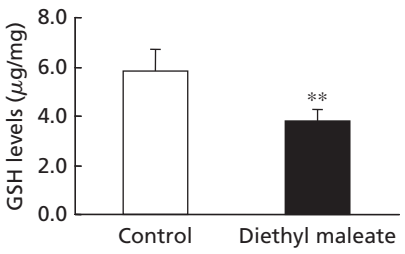

Figure 1 Brain glutathione concentration 4 h after diethyl maleate injection in rats. The data were expressed in μg/mg protein. Data are mean ± SD, $n = 8$. GSH, glutathione. $^{**}P < 0.05$ compared with control group.

Effect of glutathione depletion on P-glycoprotein expression at the blood–brain barrier in rats

Western blotting analysis (Figure 2a, b) showed that P-gp expression of brain cortex was significantly upregulated in the rats treated with DEM as compared with control rats, as indicated by summary densitometric analysis (100% in control vs 154.4 ± 14.0% in GSH-depleted group). Treatment with the antioxidant *N*-acetylcysteine attenuated upregulation of P-gp expression induced by GSH depletion in rats.

RT-PCR analysis was used to study whether mdr1a and mdr1b mRNA expression was altered at the blood–brain barrier in rats treated with DEM. Four hours after injection of rats with DEM, an increase in expression of mdr1a mRNA was detected. However, there was little change in the expression of mdr1b mRNA, which suggested that GSH-depletion induced the upregulation of mdr1a mRNA expression (Figure 3).

Effect of glutathione depletion on brain rhodamine 123 uptake in rats

The plasma and brain concentrations of Rh123 were measured 45 min after intravenous administration of Rh123 to rats. Compared with control rats, the Rh123 concentration in plasma increased significantly in GSH-depleted rats, whereas the Rh123 brain content was reduced (Table 1). Significant differences were found in the brain-to-plasma concentration ratio (Kp value) of Rh123 between the GSH depletion group and the control group (Table 1). All the data demonstrated that a low level of GSH could bring about strengthening of P-gp function at the blood–brain barrier. We also found that *N*-acetylcysteine treatment decreased the concentration of plasma Rh123 by 26% and increased brain Rh123 levels by 14% in GSH-depleted rats, and its Kp value was significantly increased (Table 1). This suggested that the antioxidant *N*-acetylcysteine would, at least partially, prevent P-gp upregulation induced by GSH depletion in rats.

Discussion

Four hours after DEM treatment in rats, the brain GSH content was significantly reduced whilst the P-gp level was significantly increased compared with control rats. The mdr1a mRNA expression at the blood–brain barrier was upregulated, whereas no significant change in mdr1b mRNA expression was found. At the same time, the Kp values of Rh123 suggested that function of P-gp was significantly enhanced at the blood–brain barrier in GSH-depleted rats. Treatment of rats with *N*-acetylcysteine reduced P-gp upregulation induced by DEM, suggesting ROS might have been involved in P-gp expression *in vivo*.

GSH is considered to be the most prevalent and important intracellular nonprotein thiol/sulfhydryl compound in mammalian cells. It plays an important role in protection against

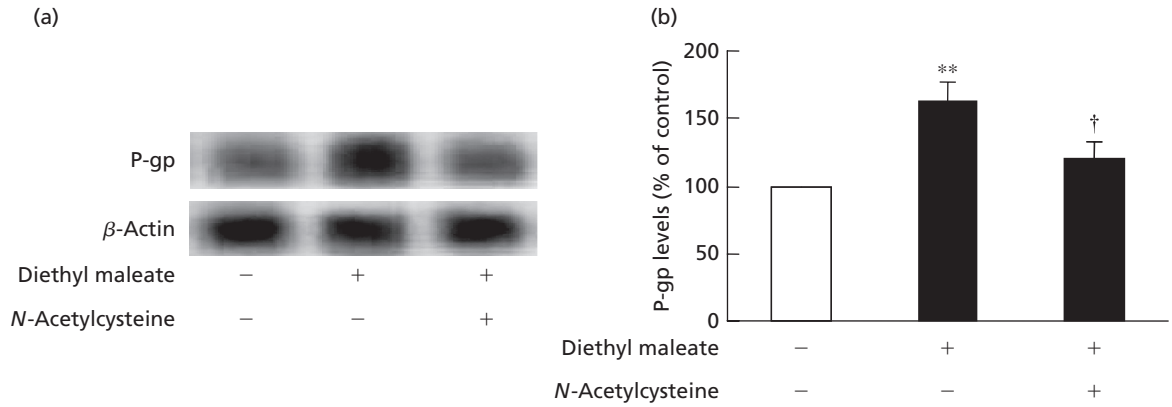

Figure 2 Effect of glutathione depletion on P-glycoprotein level at the blood–brain barrier in rats. The rats were decapitated 4 h after injection of diethyl maleate and brain microvessels were isolated for Western blotting analysis. *N*-acetylcysteine was administered (100 mg/kg, i.p.) 1 h after treatment with diethyl maleate. Representative immunoblot bands (a) and summary densitometric analysis (b) are shown (data are reported as means with SD from five rats). P-gp, P-glycoprotein. $^{**}P < 0.01$ compared with control rats; $^{\dagger}P < 0.05$ compared with glutathione-depleted rats.

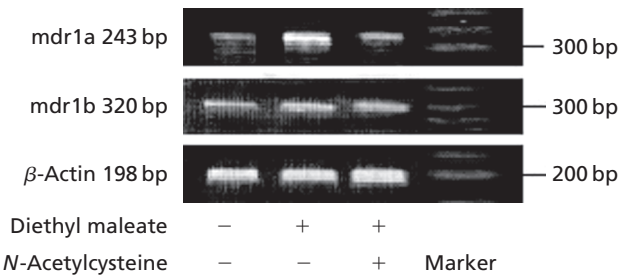

Figure 3 Relative RT-PCR analysis of mdr1a and mdr1b gene expression at the blood–brain barrier in rats after administration of diethyl maleate. Total cellular RNA was isolated from the rat brain cortex 4 h after treatment with diethyl maleate. *N*-acetylcysteine was administered (i.p.) 1 h after treatment with diethyl maleate.

oxidative stress and free radical damage via intervention of an enzyme and also via small molecules such as α-tocopherol and ascorbic acid.[28,29] Thus, GSH depletion induces an imbalance in antioxidant defence, paving the way for oxidative stress. Neurological and mental disturbances in humans with inherited defects of the GSH system indicate its involvement in central nervous system functions. An altered GSH level has also been reported in various neurological diseases.[30,31] Our studies suggested that GSH was likely to affect the transport of some drugs across the blood–brain barrier due to upregulation of P-gp expression by GSH depletion. Permeation studies performed with freshly-excised guinea-pig ileum showed that GSH not only enhanced the absorptive transport of Rh123 across intestinal tissue, but also reduced the basolateral to apical secretion of Rh123.[23] DEM is a commonly used agent for acute GSH depletion because it diffuses freely into cells due to its hydrophobicity and forms glutathione conjugates in reactions catalysed by glutathione *S*-transferase, which results in the decreased scavenging of ROS.[24,25,32] The investigations in this study were undertaken using a dose of DEM (912 mg/kg) that did not result in animal death. The experimental rats appeared to be able to tolerate this dose of DEM without any sign of damage. Increased P-gp gene expression induced by GSH depletion could be a protective response. Modulation of P-gp levels as an adaptive response to oxidative stress could conceivably be an important regulation mechanism for survival in conditions of sudden environmental change. Increased levels of P-gp provided, for example, protection against cadmium- and ROS-induced apoptosis.[18] Suggested mechanisms whereby P-gp could improve cell survival include efflux and, hence, removal of intracellular ceramides involved in initiating apoptosis.

In this study, the assay for leakage of Evans Blue dye from brain vessels showed that the blood–brain barrier permeability was not damaged 4 h after treatment with DEM (912 mg/kg) in rats. However, it was noteworthy that sustained elevation of intracellular ROS resulting from GSH depletion might have damaged membrane protein

Table 1 Effect of glutathione depletion on brain uptake of rhodamine 123 in rats

	Plasma Rh123 (ng/ml)	Brain Rh123 (ng/g)	Kp value (ml/g)
Control	69.77 ± 6.05	3.64 ± 0.17	0.052 ± 0.004
GSH depletion	137.96 ± 24.97*	2.74 ± 0.67*	0.020 ± 0.004**
GSH depletion + *N*-acetylcysteine	102.04 ± 11.96	3.13 ± 0.57	0.031 ± 0.003†

Data are expressed as means ± SD (n = 6). GSH, glutathione. Kp value, brain-to-plasma concentration ratio. Rh123, rhodamine 123. $^{*}P < 0.05$, $^{**}P < 0.01$ compared with control group. $^{\dagger}P < 0.05$ compared with GSH depletion group.

and function, even some genes.[33] Our results showed that increases in the expression and function of P-gp were markedly suppressed by a ROS scavenger, *N*-acetylcysteine, in rats, thus further confirming a redox-sensitive regulation of the resistance-related mdr1a and mdr1b isoforms.[15] This was consistent with the results of recent studies that oxidative stress increased P-gp expression in rat brain capillary endothelial cells in primary culture.[19,21]

Investigation to uncover the exact mechanisms of signal transduction for upregulation of P-gp by GSH depletion-induced P-gp expression would be interesting. Further studies are required to clarify whether enhancement of the antioxidant capacity might be an effective strategy to counteract intrinsic mdr1 overexpression *in vivo*, and to see if this might contribute to reversal of multidrug resistance.

Conclusions

We have investigated *in vivo* the effects of GSH depletion by DEM on P-gp expression at the blood–brain barrier in rats. The oxidative stress induced by GSH depletion played a positive role in the regulation of function and expression of P-gp at the blood–brain barrier in rats.

Declarations

Conflict of interest

The Author(s) declare(s) that they have no conflicts of interest to disclose.

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