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Short Communication

IN VIVO AND *IN VITRO* EVIDENCE FOR ATP-DEPENDENCY OF P-GLYCOPROTEIN-MEDIATED EFFLUX OF DOXORUBICIN AT THE BLOOD–BRAIN BARRIER

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Abstract—We investigated the role of ATP in the active efflux of doxorubicin (DOX) mediated by P-glycoprotein (P-gp), the multidrug-resistance (MDR) gene product, at the blood–brain barrier. In transient brain ischemic rats prepared with 4-vessel occlusion of vertebral and common carotid arteries for 20 min, a procedure that depleted their brain ATP content to 3% that of normal rats, the estimated permeability coefficient of DOX was increased 17-fold (to 243 ± 2.5 μL/min/g brain). When the ATP content recovered to a normal level by means of 30-min and 24-hr cerebral recirculation of blood, the permeability coefficient recovered to 14.0 ± 5.0 and 18.4 ± 2.3 μL/min/g brain (mean ± SEM, N = 3–6), respectively, very close to the control permeability (14.3 ± 1.5 μL/min/g brain). The uptake of DOX by primary cultured brain capillary endothelial cells expressing P-gp at the luminal membrane was increased significantly (up to 2-fold), which correlated well with the decrease of cellular ATP contents caused by treating the cells with metabolic inhibitors. Evidence for the ATP-dependent transport of DOX obtained from the present *in vivo* and *in vitro* studies strongly indicates that P-gp in the brain capillaries functions actively as an efflux pump in the physiological state, providing a major mechanism to restrict the transfer of DOX into the brain.

Key words: ATP-dependent transport; blood–brain barrier; doxorubicin; ischemia; P-glycoprotein; multidrug resistance

P-gp§ is a transmembrane glycoprotein expressed in MDR tumor cells and several normal tissues including liver, kidney, adrenal gland, gravid uterus, intestine and capillary endothelium in brain [1, 2]. By sequence analysis, P-gp has been classified as the ATP-binding cassette (ABC) superfamily [3], or the traffic ATPase [4]. In fact, it has been demonstrated that drug efflux mediated by P-gp is an energy-dependent process with high ATPase activity [5] and that mutagenesis of nucleotide-binding domains of P-gp induces a failure to eject drugs [6].

In normal tissues, using membrane vesicles from liver bile canaliculi and from the brush-border of the small intestine, ATP has been demonstrated to be a potent stimulator of daunomycin transport, whereas non- or slowly hydrolyzable ATP-analogs are ineffective [7, 8]. Therefore, P-gp is thought to function as an active drug efflux pump in normal tissues. Recently, by means of transport studies using an *in vitro* BBB model, we demonstrated that P-gp expressed in the luminal membrane of primary cultured bovine BCECs pumps vincristine and cyclosporin A out of the cells [9, 10]. The efflux is also dependent on metabolic energy and is inhibited by various MDR-reversing agents [9, 10], as reported in MDR tumor cells. Moreover, by using specific inhibitors of P-gp, we have shown that P-gp is very likely to function in the physiological state [11]. However, there is no direct evidence concerning how the cellular ATP in BCECs affects the transport of drugs by P-gp.

In the present study, we attempted to evaluate the significance of ATP content in the *in vivo* brain and *in vitro* primary cultured BCECs on P-gp-mediated efflux of DOX, an anticancer drug that has been well established as a substrate of P-gp in MDR tumor cell lines [12]. Until now, the observation that peripheral tissue-permeable DOX cannot enter the brain [13, 14] has been explained by the presence of a threshold of molecular weight [13]. However, we propose the hypothesis that DOX is actively pumped out of BCECs by P-gp, as verified previously for *Vinca* alkaloids [9, 15, 16], cyclosporin A [10, 11, 17], ivermectine [16] and rhodamine 123 [18]. First, we studied the ATP dependence of the transfer of DOX into the brain by using forebrain ischemia, which can induce an ATP-depletion state in the *in vivo* brain [19]. Additionally, since cellular

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§ Abbreviations: BBB, blood–brain barrier; BCECs, brain capillary endothelial cells; DOX, doxorubicin; MDR, multidrug resistance; and P-gp, P-glycoprotein.

Table 1. Effect of brain ischemia on the transport of DOX and sucrose into the brain

	Permeability coefficient (μL/min/g brain)		ATP content (μmol/g brain)
	DOX*	[^{14}C]Sucrose	
Control	14.3 ± 1.5	19.0 ± 1.0	1.43 ± 0.21
Post-ischemia			
0 min	243 ± 2.5†	20.0 ± 1.0	0.040 ± 0.017†
30 min	14.0 ± 5.0	24.0 ± 1.0	1.73 ± 0.31
24 hr	18.4 ± 2.3	23.0 ± 3.0	1.50 ± 0.090

Cerebrovascular permeability of DOX was measured at 37° for 30 sec at the perfusion rate of 4.98 mL/min, and ATP contents were measured with the luciferin-luciferase method. Each value is the mean ± SEM (N = 3–6).

* Permeability coefficient was corrected for the apparent vascular space estimated from [^{14}C]sucrose.

† Significantly different from the control as evaluated by ANOVA ($P < 0.05$).

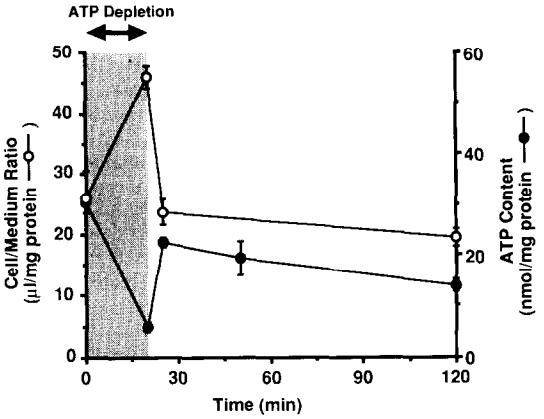

Fig. 1. Time courses for the cellular ATP content (●) and the uptake of DOX by primary cultured BCECs (○). Cellular ATP was depleted using a combination of 10 mM NaN_3, 10 mM NaF and 10 mM 3-*O*-methylglucose for 20 min (*shaded part*) and later recovered by replacing the medium with a metabolic inhibitor-free mixture containing 10 mM D-glucose. Each point represents the mean ± SEM (N = 4–6).

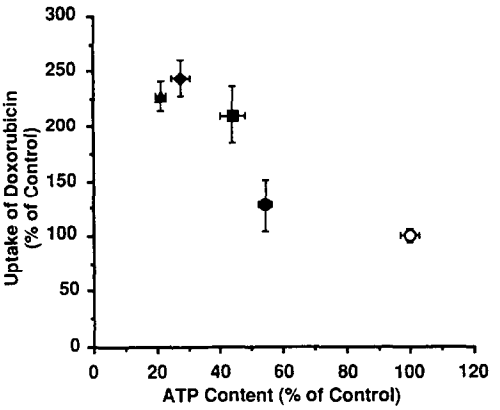

Fig. 2. Effect of cellular ATP content on the uptake of DOX by primary cultured BCECs. Cellular ATP levels were controlled by treating BCECs with various metabolic inhibitors for 20 min, and the uptake of DOX into BCECs was determined under each of the following conditions: (○) 10 mM D-glucose (ATP content was 27.8 ± 1.7 nmol/mg protein and uptake was 25.8 ± 6.0 μL/mg protein), (●) 10 mM NaN_3 and 10 mM 3-*O*-methylglucose, (■) 10 mM NaN_3, (◆) 1 mM dinitrophenol and 10 mM 3-*O*-methylglucose, and (▲) 10 mM NaN_3, 10 mM NaF and 10 mM 3-*O*-methylglucose. Each symbol represents the mean ± SEM (N = 3–6).

ATP content can be manipulated by treating cells with several metabolic inhibitors [20], we examined the effects of changes in intracellular ATP content on the *in vitro* uptake of DOX by primary cultured BCECs.

Materials and Methods

Animals. Male Fischer rats weighing 200–250 g (F344, Japan Charles River Co., Shiga, Japan) were used for the brain ischemic studies. They had free access to food and water.

Chemicals. DOX was purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan), [^{14}C-U] sucrose (5.0 mCi/mmol) from New England Nuclear (Boston, MA, U.S.A.), and ATP bioluminescence CLS (ATP measurement kit) from Boehringer Mannheim GmbH (Mannheim, Germany). All other chemicals were of reagent grade and commercially available.

Perfusion of DOX into the ATP-depleted brain. BBB permeability of DOX under ATP depletion was measured by using the internal carotid artery perfusion method [21] in 4-vessel occluded forebrain ischemic rats [22, 23], as described previously in detail [11]. Both DOX (2.0 mg/mL) and [^{14}C]sucrose (0.01 μCi/mL) were perfused at the rate of 4.98 mL/min for 30 sec. To determine the amount of DOX taken up by the brain, HPLC fluorometric analysis was performed. After homogenization of the hemisphere in a 10-fold volume of 50 mM Tris buffer (pH 7.4), a 2.0-mL aliquot of the

homogenate spiked with the internal standard (200 ng of daunomycin) was kept on ice for 10 min. Drugs were purified by extracting with 8 mL of chloroform–isopropanol (1:1) and 1.4 g of ammonium sulfate. The organic phase was evaporated, and the residue was reconstituted in methanol and applied to an HPLC system equipped with a fluorescence detector (excitation: 465 nm; emission: 580 nm). The intrinsic cerebrovascular permeability of DOX, expressed as the permeability coefficient, was obtained after correction of the apparent vascular space estimated from [^{14}C]sucrose.

Isolation and culture of BCECs. BCECs were isolated from cerebral gray matter of bovine brains as described previously [24]. Isolated BCECs were seeded on dishes that were coated with rat tail collagen and human fibronectin and cultured at 37° with 95% air and 5% CO_2. Transport experiments were performed when cells reached confluence in 10–12 days.

Transport experiment using cultured BCECs. Uptake of DOX into primary cultured monolayers of BCECs was studied by a method reported previously [24], with minor modification. Briefly, the cultured BCECs were preincubated at 37° in incubation solution (141 mM NaCl, 4 mM KCl, 2.8 mM $CaCl_2$, 0.5 mM $MgSO_4$, 10 mM HEPES, pH 7.4) containing various metabolic inhibitors or 10 mM D-glucose. Immediately after the preincubation, the solution was removed by suction, and incubation solution (250 μL) containing DOX (40 μg/mL) was added to each well to initiate transport. To terminate the transport reaction, cells were washed three times with 1 mL of ice-cold incubation solution after 2 min. Then cells were harvested with 200 μL of 0.1% trypsin–0.1% EDTA containing daunomycin (500 ng/mL) as an internal standard and were homogenized by Ultra-turrax. After extraction with 800 μL acetonitrile, DOX was measured with fluorescence HPLC. Protein content in cultured cells was determined by the method of Lowry *et al.* [25], using BSA as a standard. The uptake was expressed as the cell-to-medium concentration ratio (μL/mg protein) by correcting for an extracellularly adsorbed amount estimated from the uptake of DOX for 10 sec at 4°.

ATP determination. ATP contents of the brain and primary cultured BCECs were measured by the luciferin-luciferase assay [26] using the ATP bioluminescence CLS kit. The brain was frozen *in situ* by liquid nitrogen applied on the scalped head, weighed, and homogenized in 3.0 M $HClO_4$. BCECs were harvested with a rubber policeman in 250 μL of 2% $HClO_4$-2 mM EDTA solution. The mixture was centrifuged, and the supernatant was neutralized with KOH. The mixture was centrifuged again, and the supernatant was stored at −80° until analyzed. The photons were measured by a liquid scintillation counter.

Results and discussion

When ischemia is produced by the 4-vessel occlusion technique in rats, ATP content in the brain decreases markedly to a few percent of that of normal rats [20]. As shown in Table 1, the ATP content in the brains of control rats was 1.43 ± 0.21 μmol/g brain, a finding similar to that of a previous report [19]. ATP was severely depleted to only 3% of the control level (0.040 ± 0.017 μmol/g brain) by 20-min ischemia. After 30 min and 24 hr of cerebral recirculation of blood, ATP contents in the brain had recovered to the control level. Interestingly, as also shown in Table 1, the change in the permeability coefficient of DOX inversely corresponded to the change in ATP contents, whereas the permeability of [^{14}C]sucrose was not altered. These results strongly suggest that an ATP-dependent mechanism, i.e. P-gp, prevents the penetration of DOX into the brain in the physiological state.

Since the ATP content in the whole brain does not necessarily represent that in BCECs, further studies using *in vitro* primary cultured BCECs were undertaken to determine whether the effects of ATP on the transport of DOX, as shown in Table 1, were reproducible in cultured cells. As shown in Fig. 1, when ATP in BCECs was decreased to 20% by treating with a combination of 10 mM NaN_3, 10 mM NaF and 10 mM 3-*O*-methylglucose for 20 min, the uptake of DOX by BCECs increased approximately 2-fold. Moreover, by replacing the medium with a metabolic inhibitor-free incubation mixture containing 10 mM D-glucose, the uptake of DOX was reversed rapidly to the control level with the recovering intracellular ATP contents. These results indicate that the uptake of DOX by BCECs is dependent upon the ATP content in cells and is consistent with the results obtained by the transient brain ATP depletion model (Table 1).

How much cellular ATP content does P-gp need to pump DOX out of BCECs? It has been reported that the treatment of cells with various metabolic inhibitors results in various levels of cellular ATP [20]. In BCECs, as shown in Fig. 2, we could induce various cellular ATP levels, of which the lowest value was approximately 20% of control. The uptake of DOX by BCECs exhibited a threshold response to changes in the cellular ATP content (Fig. 2). When ATP content decreased to about 40% of total cellular ATP, the uptake of DOX was increased remarkably, and it was suggested that P-gp expressed in BCECs apparently required at least 50% of total cellular ATP to function as a drug efflux pump.

P-gp is a substrate-stimulated ATPase, and P-gp-mediated efflux of drugs is an energy-dependent process in MDR tumor cells and in normal small intestine [7] and liver [8]. In the present study, it was confirmed that DOX is transported by P-gp expressed at the brain capillary in the physiological state and that P-gp transferred DOX out of cells in an ATP-dependent manner. Furthermore, the efflux activity of DOX at the BBB was correlated with ATP content in the brain, especially in BCECs. It has been demonstrated recently that P-gp expressed on the luminal surface of BCECs can pump various drugs, including vincristine, cyclosporin A and rhodamine 123, out of the cells [9–11, 15, 17, 18]. Moreover, the recent finding that a disruption of *mdr1a* gene in mouse leads to a remarkably increased accumulation of vinblastine and ivermectin in the brain [16] strongly supports the previous findings and the present results. From these results, it was

clarified that P-gp functions actively as the BBB to restrict the permeation of cytotoxic compounds.

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