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Effect of bioflavonoids on vincristine transport across blood–brain barrier[1]

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Abstract

Several grapefruit juice bioflavonoids, including quercetin, are reported to stimulate P-glycoprotein-mediated drug efflux from cultured tumor cells. To see whether these bioflavonoids alter the permeation of vincristine across the blood–brain barrier, we conducted experiments with cultured mouse brain capillary endothelial cells (MBEC4 cells) in vitro and ddY mice in vivo. The steady-state uptake of [^{3}H]vincristine by MBEC4 cells was decreased by 10 μM quercetin, but increased by 50 μM quercetin. Similarly, the in vivo brain-to-plasma concentration ratio of [^{3}H]vincristine in ddY mice was decreased by coadministration of 0.1 mg/kg quercetin, but increased by 1.0 mg/kg quercetin. Kaempferol had a similar biphasic effect on the in vitro uptake of [^{3}H]vincristine. Other aglycones tested (chrysin, flavon, hesperetin, naringenin) increased [^{3}H]vincristine uptake in the 10–50 μM range, and glycosides (hesperidin, naringin, rutin) were without effect. We then addressed the mechanism of the concentration-dependent biphasic action of quercetin. Verapamil, a P-glycoprotein inhibitor, inhibited the efflux of [^{3}H]vincristine from MBEC4 cells, while 10 μM quercetin significantly stimulated it. The uptake of [^{3}H]vincristine by MBEC4 cells was increased by inhibitors of protein kinase C, but decreased by phorbol 12-myristate-13-acetate (PMA), as well as by 10 μM quercetin. The phosphorylation level of P-glycoprotein was increased in the presence of 5 μM quercetin or 100 nM PMA, but decreased by the protein kinase C inhibitor H7 (1-(5-isoquinolinesulfonyl)-2-methylpiperazine, 30 μM). We conclude that low concentrations of quercetin indirectly activate the transport of [^{3}H]vincristine by enhancing the phosphorylation (and hence activity) of P-glycoprotein, whereas high concentrations of quercetin inhibit P-glycoprotein. Our results indicate that patients taking drugs which are P-glycoprotein substrates may need to restrict their intake of bioflavonoid-containing foods and beverages, such as grapefruit juice.

Keywords: P-Glycoprotein; Bioflavonoid; Blood–brain barrier; Vincristine; Protein kinase C

1. Introduction

Large amounts of bioflavonoids are ingested because of their abundance and wide distribution in foods and beverages. Recently, it has been reported that co-administration of grapefruit juice with various drugs led to an increase in the plasma concentration of the drugs, and these drug–grapefruit juice interactions may be caused by bioflavonoids (Bailey et al., 1991, 1993, 1995; Benton et al., 1994; Ducharme et al., 1995; Kupferschmidt et al., 1995). For example, the bioavailability of dihydropyridine Ca^{2+} channel blockers (felodipine, nifedipine, nicardipine and manidipine), an immunosuppressive agent (cyclosporine A), a benzodiazepine narcoleptic (midazolam) and an antiallergic agent (terfenadine) was significantly increased by the co-administration of grapefruit juice (Bailey et al., 1991, 1993, 1995; Benton et al., 1994; Ducharme et al., 1995; Kupferschmidt et al., 1995). Among several possible mechanisms for the above interactions such as enhanced absorption, altered plasma protein binding or impaired hepatic metabolism by grapefruit juice, impaired first-pass metabolism due to the inhibition of the intestinal enzyme CYP (cytochrome *P*450)-3A was considered the most plausible.

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P-glycoprotein actively pumps out various drugs, such as vincristine and digoxin, from the intracellular space to the outside (Juliano and Ling, 1976; Kartner et al., 1983). This protein is expressed in various multidrug-resistant tumor cell membranes, but also in normal tissues such as brain capillary endothelial cells, the adrenal cortex, the brush-border membrane of renal proximal tubules, the apical membrane of the colon and jejunum, the bile canalicular membrane of the liver, etc. (Thiebau et al., 1987; Bradley et al., 1990). P-glycoprotein expressed on brain capillary endothelial cells is a key component of the blood–brain barrier (Croop et al., 1989; Schinkel et al., 1994; Ohnishi et al, 1995). Recently, Phang et al. reported that some bioflavonoids, such as kaempferol, galangin and quercetin, stimulate P-glycoprotein-mediated drug efflux in tumor cells derived from human mammary adenocarcinoma (MCF-7 cells) or cultured human colon tumor cells (HCT-15) (Phang et al., 1993; Critchfield et al., 1994). Because many substrates or inhibitors of P-glycoprotein are metabolized by CYP3A4 (Zhou-Pan et al., 1993; Zhou et al., 1993; Wacher et al., 1995), it is thought that the drug-grapefruit juice interactions mentioned above may be associated in part with the functional alteration of P-glycoprotein by the bioflavonoids in grapefruit juice.

In this study, we investigated the effects of various bioflavonoids on the blood–brain barrier transport of vincristine, using both in vitro transport experiments with cultured mouse brain capillary endothelial cells (MBEC4 cells) (Tatsuta et al., 1992, 1994) and in vivo brain distribution experiments in mice.

2. Materials and methods

2.1. Regents and animals

Quercetin, naringin, naringenin, hesperetin, hesperidin, flavon, chrysin, rutin, phorbol 12-myristate-13-acetate (PMA) and staurosporine were purchased from Sigma (St. Louis, MO, USA). Fisetin was purchased from Aldrich Chemical (Milwaukee, WI, USA). Verapamil, K252a (9,1-epoxy-1H-diindolo(1,2,3-*fg*:3′,2′,1′-*kl*)pyrrolo(3,4-*i*)(1,6)benzodiazocine-10-carboxylic acid,2,3,9,10,11,12-hexahydro-10-hydroxy-9-methyl-1-oxo-, methyl ester, (9-α,10-β,12-α)-) and Ro31-8220 (3-[1-[3-(amidinothio)propyl]-3-indolyl]-4-(1-methyl-3-indolyl)-1*H*-pyrrole-2,5-dionemethanesulfonate) were purchased from Nacalai Tesque (Kyoto, Japan). 1-(5-Isoquinolinesulfonyl)-2-methylpiperazine (H-7) and protein kinase C inhibitor peptide (19–31) were purchased from Seikagaku (Tokyo, Japan). [^{3}H]Vincristine (8.7 Ci/mmol), [^{14}C]3-*O*-methylglucose (11 mCi/mmol), [^{14}C]phenylalanine (448 mCi/mmol) and ^{32}Pi (10 mCi/mmol) were purchased from Amersham International (Buckinghamshire, UK). The C219 monoclonal antibody was purchased from Centocor (Malvern, PA, USA). Anti-*mdr* serum was purchased from Oncogene Research Products (Cambridge, MA, USA). Alkaline phosphatase-conjugated goat anti-rabbit immunoglobulin G (IgG) was purchased from Organon Teknika (West Chester, PA, USA). *p*-Nitrophenyl phosphate was purchased from Life Technologies (Gaithersburg, MD, USA). All other chemicals used in the experiments were of reagent grade.

The ddY mice (20–25 g) were purchased from Seac Yoshitomi (Fukuoka, Japan).

2.2. Cell culture

Cells of the MBEC4 cell line were cultured in Dulbecco's modified Eagle's medium (Nikken Bio Medical Laboratory, Kyoto, Japan) supplemented with 10% fetal calf serum, 100 units/ml penicillin G and 100 μg/ml streptomycin under the conditions of 37°C and 5% CO_2/95% air (Tatsuta et al., 1992). For the uptake study, the cells were seeded at 4×10^4 cells/ml in a well (four-well multidish, Nunc, Denmark). The cells were grown for 3 days and used for the uptake study.

2.3. Cellular uptake of [^{3}H]vincristine by MBEC4 cells

The culture medium was removed and cells were washed twice with 1 ml of incubation buffer (141 mM NaCl, 4 mM KCl, 2.8 mM $CaCl_2$, 1 mM $MgSO_4$, 10 mM D-glucose, 10 mM HEPES; pH 7.4) at 37°C. Uptake experiments were performed in incubation buffer containing 30 nM [^{3}H]vincristine in the presence or absence of bioflavonoids, verapamil, protein kinase C inhibitors or PMA at 37°C. The cells were washed three times with 1 ml of ice-cold buffer to stop uptake. Cells were dissolved with 3 N NaOH (200 μl), then neutralized with 6 N HCl (100 μl), and scintillation cocktail (Clear-sol I, Nakalai Tesque, Kyoto, Japan) was added. The radioactivity of intracellular [^{3}H]vincristine was then determined with a liquid scintillation counter (LS6500, Beckman Instruments, Fullerton, CA, USA). The amount of protein in the cells was measured by Lowry's method (Lowry et al., 1951). The uptake of [^{3}H]vincristine is expressed as uptake per mg protein of the cells relative to the drug concentration (μl/mg protein).

2.4. Efflux of [^{3}H]vincristine from MBEC4 cells

Cells were preincubated with the medium containing 100 nM [^{3}H]vincristine in the presence or absence of 10 μM quercetin or 10 μM verapamil for 60 min. The incubation medium was then replaced by fresh medium with or without 10 μM quercetin or 10 μM verapamil and the cells were incubated for appropriate times. The cells were washed twice with 1 ml of ice-cold buffer to stop the efflux of [^{3}H]vincristine. The radioactivity of intracellular [^{3}H]vincristine was measured by the method described for the uptake experiment.

2.5. In vivo plasma / brain distribution of [^{3}H]vincristine

[^{3}H]Vincristine (1.0 mg/kg or 1.5 mg/kg) was administered with or without quercetin (0.1 mg/kg or 1.0 mg/kg) into the tail vein of ddY mice. Brain and plasma samples were taken at 30 min after intravenous administration of [^{3}H]vincristine. Brain samples were dissolved in 1.5 ml of Solvable (Packard, Meriden, CT, USA), and then neutralized. For the plasma sample (50 μl), 2 N KOH–isopropanol solution (0.2 ml) was added and the mixture was neutralized with 10% acetic acid (0.4 ml). The radioactivity of the brain or plasma samples was measured with a liquid scintillation counter. The K_p value of [^{3}H]vincristine was obtained from the brain-to-plasma concentration ratio.

2.6. [^{32}P]Labeling and immunoprecipitation of P-glycoprotein

[^{32}P]Labeling and immunoprecipitation of P-glycoprotein were done as described previously (Chambers et al., 1990). Cultured cells were incubated with medium containing ^{32}Pi (200 μCi/ml) in the presence or absence of quercetin (0–50 μM), 30 μM H-7 and 100 nM PMA for 60 min in 5% CO_2/95% air at 37°C. After incubation, the cells were washed three times with 1 ml of ice-cold buffer, dissolved in 1 ml of cell lysis buffer (50 mM Tris–HCl, 140 mM NaCl, 5 mM NaF, 2 mM sodium vanadate, 0.2 mM phenylmethylsulfonyl fluoride, 25 μg/ml aprotinin, 4 mM EDTA, and 0.5% sodium deoxycholate at pH 8.0) and centrifuged at 15,000 rpm for 30 min. P-glycoprotein was then immunoprecipitated by C219 monoclonal antibody from cell extracts. In the immunoprecipitation experiments, 50 μl of Protein A-Sepharose FF (Pharmacia Biotech, Uppsala, Sweden) was added to the cell extracts in order to remove the proteins nonspecifically binding to IgG antibody and the mixture was mixed with a minidisk rotor at 4°C for 60 min. After centrifugation at 5000 rpm for 1 min, the supernatants were collected, mixed with 100 μg/ml of C219 monoclonal antibody (100 μl), and allowed to stand at 4°C for 60 min. Protein A-Sepharose FF (100 μl) was then added and mixed with a minidisk rotor at 4°C for 60 min. The ^{32}P-labeled sample was size-fractionated by electrophoresis on a 7.5% sodium dodecyl sulfate (SDS)-polyacrylamide gel. Labeled samples were analyzed by autoradiography. To quantitate the relative amounts of labeled P-glycoprotein in each gel, autoradiograms of ^{32}P-labeled P-glycoprotein were scanned on an imaging analyzer (BAS 2000, FUJIX, Tokyo, Japan).

2.7. Quantification of P-glycoprotein by ELISA

The ELISA procedure was described previously (Tatsuta et al., 1994). MBEC4 cells (6.0×10^2 cells in a well) were seeded in a 96-well plate. Three days after seeding, each well was washed three times with 300 μl of phosphate-buffered saline and the MBEC4 cells were treated with 200 μl of quercetin (1–50 μM) for 60 min. The cells were then washed three times with 300 μl of phosphate-buffered saline, fixed with 100 μl of 10% formalin neutral buffer solution (pH 7.4) for 4 h at room temperature, washed three times with 300 μl of phosphate-buffered saline, and incubated with 200 μl of RPMI 1640 containing 100 mM glycine, 1% bovine serum albumin, and 0.05% NaN_3 (blocking solution) for 12 h at room temperature. After aspirating the blocking solution, we treated the wells with 60 μl of the first antibody solution (phosphate-buffered saline containing 3 μg/ml of anti-*mdr* serum and 3% bovine serum albumin) for 2 h at room temperature. The wells were washed five times with 300 μl of phosphate-buffered saline and treated with 100 μl of the second antibody solution (alkaline phosphatase-conjugated goat anti-rabbit IgG at 1:12,500 dilution in phosphate-buffered saline containing 3% bovine serum albumin) for 60 min at room temperature. The wells were washed five times with 300 μl of phosphate-buffered saline, and then we added 100 μl of substrate solution containing 4.0 mg/ml of p-nitrophenyl phosphate, 10% (v/v) diethanolamine, and 0.5 mM $MgCl_2$ (pH 9.8). After incubation at room temperature for 60 min, absorbance at 405 nm in each well was measured with a micro plate reader (Model 450, Biorad, Tokyo, Japan). The absorbance of the wells without the first antibody in each group was subtracted as the background.

2.8. HPLC analysis for determination of quercetin in plasma

Quercetin was determined by high-performance liquid chromatography (HPLC). For HPLC analysis we injected 50 μl of the extracted sample onto a COSMOSIL 5C18-AR column (4.6 × 150 mm, 5 μm; Nacalai Tesque, Kyoto, Japan) with acetonitrile/methanol/0.025 M phosphate buffer pH 2.4, 8.75/33.25/58 (v/v/v) as the mobile phase at a flow rate of 1 ml/min. Chromatograms were recorded at 370 nm.

Standards were prepared with drug-free mouse plasma to give final concentrations of 0.01, 0.02, 0.05, 0.1, 0.5, 1 and 5 μg/ml of quercetin. Standard curves were generated following extraction, as described below.

2.9. Solid-phase extraction procedure

Plasma sample extraction was as described previously (Liu et al., 1995). Samples of mouse plasma (800 μl) were diluted and acidified with sodium phosphate buffer (pH 2.0, 0.33 M, 4 ml). Fisetin (60 μl, 1 μg/ml methanol solution) was added as an internal standard. Sep-Pak cartridges (Waters, Watford, UK) were pre-equilibrated with methanol (2 ml) and conditioned using sodium phosphate buffer (as above; 6 ml). The diluted plasma samples were

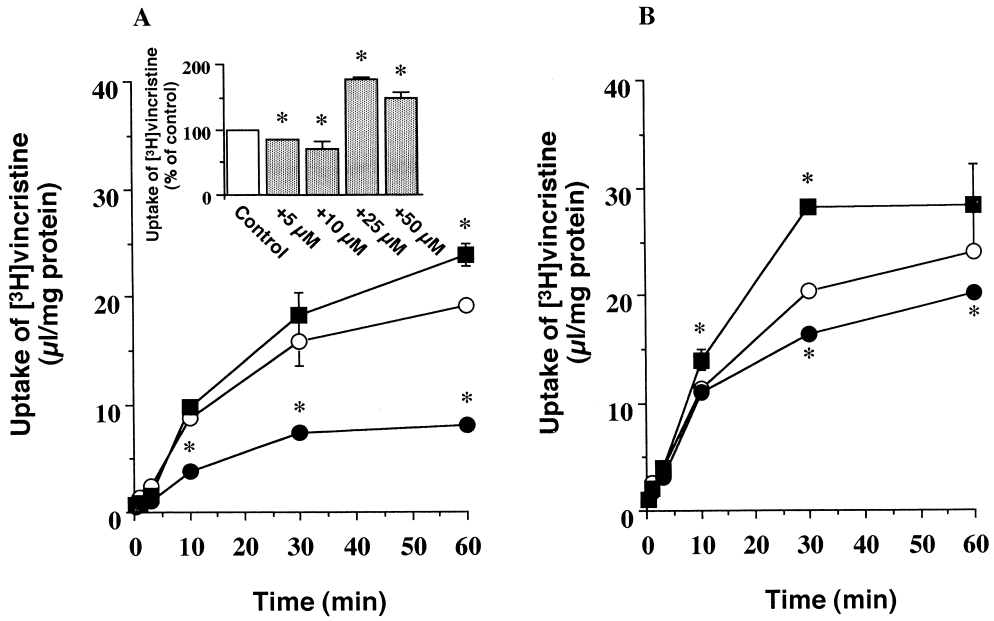

Fig. 1. Effect of quercetin (panel A) or kaempferol (panel B) on the uptake of [^{3}H]vincristine by MBEC4 cells. The uptake of [^{3}H]vincristine (30 nM) by MBEC4 cells at 37°C was measured as described in Section 2 in the presence or absence of quercetin or kaempferol. Each value represents the mean ± SE ($n = 3$–4). Significant differences were determined by Student's *t*-test ($^*P < 0.05$). (A) ○: control (0.1 % DMSO), ●: with 10 μM quercetin, ■: with 50 μM quercetin, (B) ○: control (0.1 % DMSO), ●: with 10 μM kaempferol, ■: with 50 μM kaempferol. Inset: Effect of quercetin on the uptake of [^{3}H]vincristine by MBEC4 cells at 60 min. Each value represents the mean ± SE ($n = 4$–8). Significant differences were determined by Student's *t*-test ($^*P < 0.05$).

slowly loaded onto the cartridges. Following a wash with sodium phosphate buffer (as above; 1.6 ml), quercetin and fisetin were eluted with aqueous acetone (3.2 ml, 50% v/v acetone, containing 250 mM NaH_2PO_4 and 0.1% v/v diethylamine, adjusted to pH 3.5 with Pi), dried in N_2 and redissolved in aqueous acetone (300 μl of 45% v/v acetone in 250 mM NaH_2PO_4, then adjusted to pH 3.5 with Pi). The increased osmolarity due to salts in the pellet promoted phase separation of the aqueous acetone, with bioflavonoids remaining in the water-saturated acetone layer. Following centrifugation (13,200 × *g*, 5 min), the acetone layer was used directly for HPLC analysis (sample size 50 μl).

2.10. Statistical analysis

All of the data are expressed as means ± SE Statistical analysis was performed by using Student's *t*-test. The differences between means were considered to be significant when *P* values were less then 0.05.

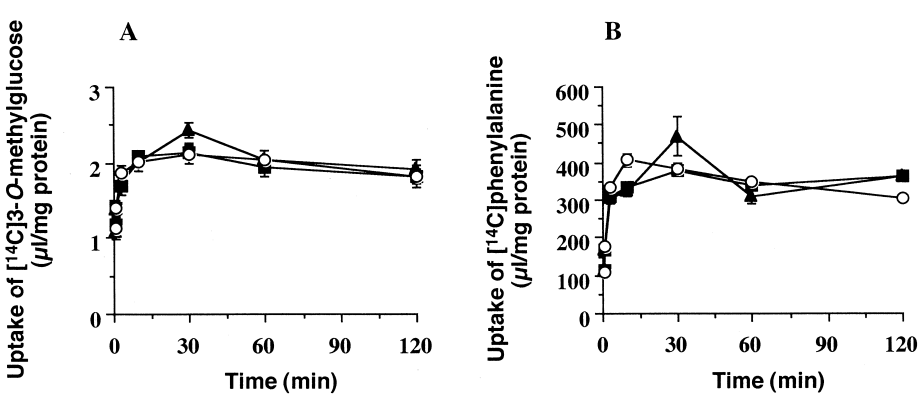

Fig. 2. Effect of quercetin or kaempferol on the uptake of [^{14}C]3-*O*-methylglucose (panel A) and [^{14}C]phenylalanine (panel B) by MBEC4 cells. The uptake of [^{14}C]3-*O*-methylglucose (1 μM) and [^{14}C]phenylalanine (500 nM) by MBEC4 cells at 37°C was measured as described in Section 2 in the presence or absence of quercetin (10 μM) or kaempferol (10 μM). Each point represents the mean ± SE ($n = 3$–4). Significant differences were determined by Student's *t*-test ($^*P < 0.05$). ○: control (0.1% DMSO), ■: with quercetin, ▲: with kaempferol.

3. Results

3.1. Effect of quercetin or kaempferol on the uptake of [^{3}H]vincristine

Fig. 1 shows the time profiles of the uptake of [^{3}H]vincristine by MBEC4 cells in the presence or absence of quercetin (panel A) or kaempferol (panel B). The uptake-time profile of [^{3}H]vincristine reached steady-state at 60 min after initiation of incubation. The uptake of [^{3}H]vincristine was significantly decreased by 10 μM quercetin or kaempferol at the steady state, while 50 μM quercetin or kaempferol increased it. The inset in Fig. 1 shows the concentration-dependent biphasic effect of quercetin. The uptake of [^{3}H]vincristine by MBEC4 cells at 60 min was significantly decreased in the presence of 5 μM or 10 μM quercetin but was increased in the presence of 25 or 50 μM quercetin.

3.2. Effect of quercetin and kaempferol on the uptake of [^{14}C]3-O-methylglucose and [^{14}C]phenylalanine

Fig. 2 shows the time profiles of [^{14}C]3-*O*-methylglucose uptake (panel A) and [^{14}C]phenylalanine uptake (panel B) by MBEC4 cells in the presence or absence (control) of 10 μM quercetin or kaempferol. There was no change in the uptake of these compounds in the presence of quercetin or kaempferol. The initial rate of [^{14}C]3-*O*-methylglucose or [^{14}C]phenylalanine uptake was not changed in the presence of 10 mM quercetin or kaempferol ([^{14}C]3-*O*-methylglucose: control, 0.53; with quercetin, 0.43; with kaempferol, 0.56 μl/mg protein/min. [^{14}C]phenylalanine: control, 133.2; with quercetin, 113.4; with kaempferol, 110.0 μl/mg protein/min).

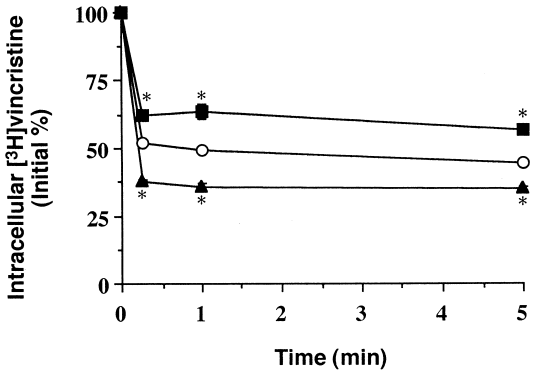

Fig. 4. Efflux of [^{3}H]vincristine from MBEC4 cells in the presence or absence of quercetin or verapamil. Cells were preincubated in growth medium containing [^{3}H]vincristine (100 nM) in the presence or absence of quercetin (10 μM) or verapamil (10 μM) for 60 min. The incubation medium was then aspirated and cells were incubated in growth medium (1 ml) with or without quercetin (10 μM) or verapamil (10 μM). At intervals, the amount of [^{3}H]vincristine retained in the cells was determined as described in Section 2. Each point represents the mean ± SE ($n = 3$–4). Significant differences were determined by Student's *t*-test ($^*P < 0.05$). ○: control (0.1% DMSO), ▲: with quercetin, ■: with verapamil.

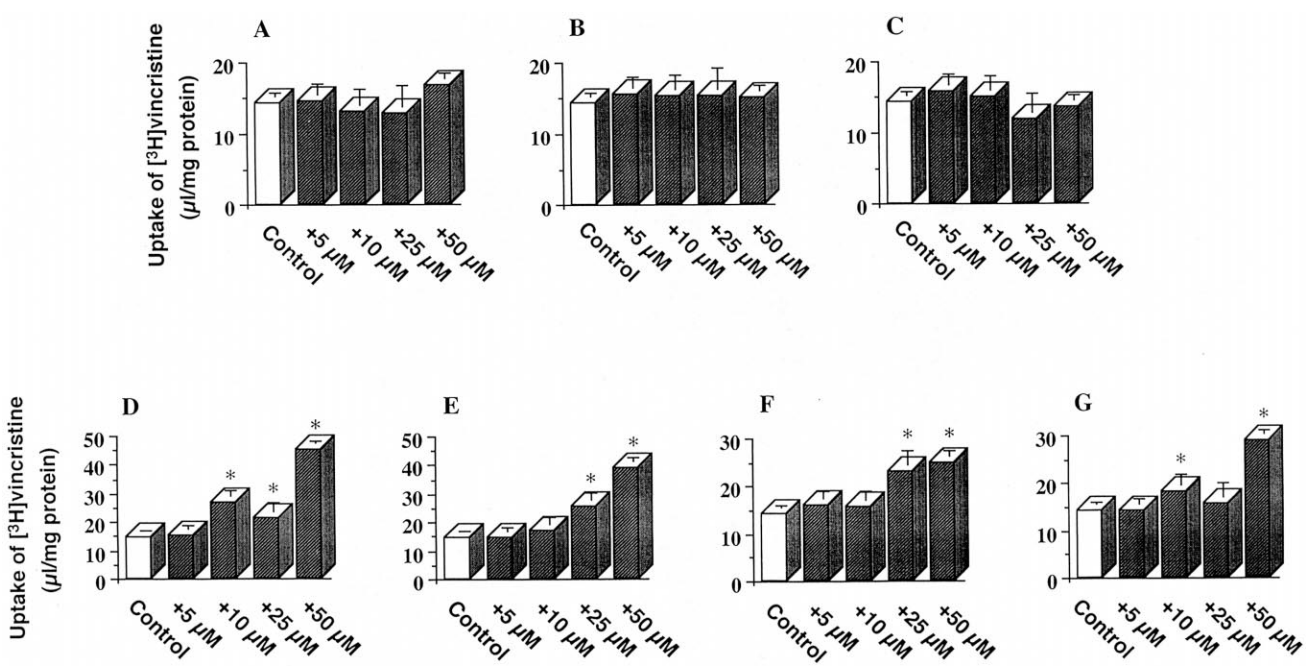

Fig. 3. Effect of various bioflavonoids, hesperidin (panel A), naringin (panel B), rutin (panel C), chrysin (panel D), flavon (panel E), hesperetin (panel F) or naringenin (panel G), on the uptake of [^{3}H]vincristine by MBEC4 cells. The uptake of [^{3}H]vincristine (30 nM) by MBEC4 cells at 37°C at 60 min was measured as described in Section 2 in the presence or absence of various concentrations of bioflavonoids (5, 10, 25, 50 μM). Each value represents the mean ± SE ($n = 3$–4). Significant differences were determined by Student's *t*-test ($^*P < 0.05$).

Table 1
Plasma and brain concentration and K_p value of [^{3}H]vincristine in the presence or absence of quercetin
[^{3}H]Vincristine (1.0 or 1.5 mg/kg) was administered with or without quercetin (0.1 or 1.0 mg/kg) into the tail vein of ddY mice. Brain and plasma samples were taken at 30 min after intravenous administration of [^{3}H]vincristine. The K_p value of [^{3}H]vincristine was obtained from the brain-to-plasma concentration ratio.
Each value represents the mean ± SE ($n = 3$). Significant differences from the values obtained after the administration of 1.0 mg/kg vincristine without quercetin were determined by the Student's *t*-test.

Vincristine (mg/kg)	Quercetin (mg/kg)	Plasma conc. (ng/ml)	Brain conc. (ng/g brain)	K_p (ml/g brain)
1.0	0	89.04 ± 4.08	17.66 ± 1.45	0.198 ± 0.013
1.5	0	221.7 ± 30.6[a]	39.71 ± 3.80[b]	0.182 ± 0.012
1.0	0.1	97.24 ± 4.45	12.42 ± 0.88[a]	0.128 ± 0.005[b]
1.0	1.0	114.2 ± 1.74[b]	31.23 ± 1.56[a]	0.274 ± 0.018[a]

[a] $P < 0.05$.
[b] $P < 0.01$.

3.3. Effect of various bioflavonoids on the uptake of [^{3}H]vincristine

Fig. 3 shows the effect of various bioflavonoids on the uptake of [^{3}H]vincristine. There was no effect of glycosides, such as hesperidin (panel A), naringin (panel B) and rutin (panel C), on the uptake of [^{3}H]vincristine, but the uptake increased in the presence of the aglycones (50 μM), such as chrysin (panel D), flavon (panel E), hesperetin (panel F) and naringenin (panel G).

3.4. Effect of quercetin on the efflux of [^{3}H]vincristine

Fig. 4 shows the effect of quercetin or verapamil on the efflux of [^{3}H]vincristine from 100 nM [^{3}H]vincristine-preloaded MBEC4 cells for 60 min. Verapamil (10 μM) significantly inhibited the efflux of [^{3}H]vincristine, while quercetin (10 μM) significantly stimulated the efflux of [^{3}H]vincristine.

3.5. Effect of quercetin co-administration on the brain distribution of [^{3}H]vincristine in mice

Table 1 shows the plasma and brain concentrations and the brain to plasma concentration ratio (K_p value) of [^{3}H]vincristine at 30 min after intravenous administration to mice with or without quercetin. The brain concentration of [^{3}H]vincristine after administration of 0.1 mg/kg quercetin was significantly reduced compared with that of the control, while there was no change in the plasma concentration. The K_p value of [^{3}H]vincristine after administration of 0.1 mg/kg quercetin was significantly lower than that of the control. However, the K_p value after administration of 1.0 mg/kg quercetin was significantly increased. This phenomenon may be due to saturation of the efflux transport process due to the high plasma concentration of [^{3}H]vincristine. Therefore, we re-examined the K_p value of [^{3}H]vincristine after the administration of 1.5 mg/kg [^{3}H]vincristine without quercetin. There was no difference in the K_p values of [^{3}H]vincristine with low and high concentrations of vincristine in plasma.

3.6. Effect of inhibitors and activator of protein kinase C on the uptake of [^{3}H]vincristine by MBEC4 cells

As shown in Fig. 5A, the uptake of [^{3}H]vincristine by MBEC4 cells was significantly increased in the presence

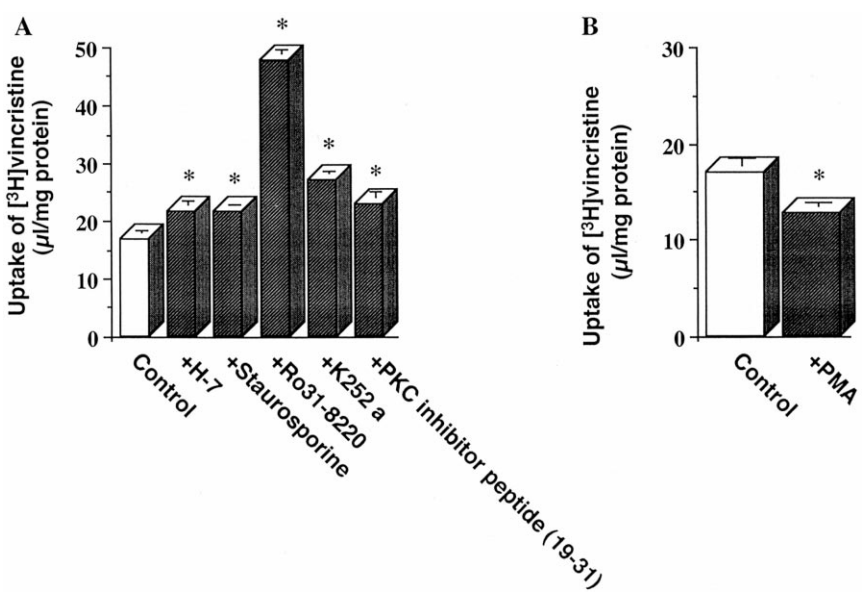

Fig. 5. Effect of inhibitors of protein kinase C, H-7 (30 μM), staurosporine (1 μM), Ro31-8220 (10 μM), K252a (10 μM) and protein kinase C inhibitor peptide (19–31) (10 μM) (panel A), or PMA (100 nM) (panel B), on the uptake of [^{3}H]vincristine by MBEC4 cells. The uptake of [^{3}H]vincristine (30 nM) by MBEC4 cells at 37°C was measured as described in Section 2 in the presence or absence of the inhibitors of protein kinase C or PMA. Each value represents the mean ± SE ($n = 3$–4). Significant differences were determined by Student's *t*-test ($^*P < 0.05$).

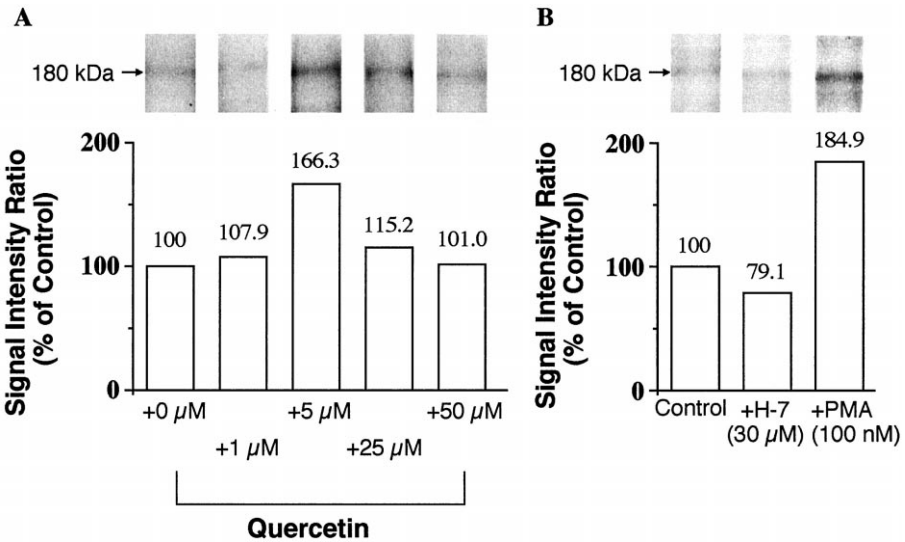

Fig. 6. Effect of quercetin (panel A), H-7 and PMA (panel B) on the phosphorylation of P-glycoprotein. ^{32}Pi was added to a final concentration of 200 μCi/ml in the absence or presence of quercetin, H-7 or PMA. MBEC4 cells were incubated at 37°C in a CO_2 incubator for 60 min. The ^{32}P-label radioactivity of P-glycoprotein was analyzed after electrophoresis on a SDS/7.5% polyacrylamide gel.

of inhibitors of protein kinase C, such as H-7 (30 μM), staurosporine (1 μM), Ro31-8220 (10 μM), K252a (10 μM) and protein kinase C inhibitor peptide (19–31) (10 μM), whereas it was significantly decreased by PMA (100 nM), an activator of protein kinase C (Fig. 5B).

3.7. Effect of quercetin or protein kinase C modulators on ^{32}P-labeling of P-glycoprotein

Fig. 6A shows the effect of quercetin on the ^{32}P-labeling of P-glycoprotein in MBEC4 cells. An increase in the phosphorylation of P-glycoprotein was observed in the presence of 5 μM quercetin. However, there was no change in the phosphorylation of P-glycoprotein at a high concentration (25, 50 μM) or low concentration (1 μM) of quercetin. PMA increased the phosphorylation of P-glycoprotein, while the phosphorylation of P-glycoprotein was reduced in the presence of H-7 (Fig. 6B).

3.8. Effect of quercetin on P-glycoprotein expression

We examined the effect of quercetin on P-glycoprotein expression in MBEC4 cells (data not shown). There was no change in P-glycoprotein expression in the presence of 1–25 μM quercetin, though it was significantly decreased in the presence of 50 μM quercetin.

4. Discussion

In this study, we found a concentration-dependent biphasic effect of quercetin on the uptake of [^{3}H]vincristine, a substrate of P-glycoprotein, in MBEC4 cells (Fig. 1): the efflux of [^{3}H]vincristine was activated by 10 μM quercetin (Fig. 4), but inhibited by 50 μM quercetin. Although the direct effect of 50 μM quercetin on the efflux of [^{3}H]vincristine was not examined, the initial uptake rate was not changed in the presence of 50 μM quercetin (control, 0.885 ± 0.045 μl/mg protein/30 s; with 50 μM quercetin, 0.726 ± 0.049 μl/mg protein/30 s), as shown in Fig. 1. Therefore, it is considered that the increased uptake of [^{3}H]vincristine by MBEC4 cells is caused by the inhibition of efflux. Quercetin did not affect the uptake of [^{14}C]3-*O*-methylglucose or [^{14}C]phenylalanine (Fig. 2). 3-*O*-Methylglucose is transported through the blood–brain barrier via facilitated diffusion in a sodium-independent manner (Pardridge, 1983), and phenylalanine is transported via the L-system in a sodium-independent manner (Pardridge, 1986). These findings indicate that quercetin did not affect the glucose and amino acid transporters in MBEC4 cells, suggesting the specific inhibition of the P-glycoprotein-mediated efflux of [^{3}H]vincristine from MBEC4 cells by quercetin. Other bioflavonoids such as hesperidin, naringin and rutin did not affect the uptake of [^{3}H]vincristine (Fig. 3A–C). At high concentration the aglycones, chrysin, flavon, hesperetin and naringenin, significantly increased the uptake of [^{3}H]vincristine (Fig. 3D–G). These findings indicate that the aglycone-type bioflavonoids modify the blood–brain barrier transport of [^{3}H]vincristine. It is considered that the increased uptake of [^{3}H]vincristine was due to inhibition of P-glycoprotein by high concentrations of bioflavonoids other than glycosides.

It is generally accepted that phosphorylation of P-glycoprotein by protein kinase C is a determinant of the transport activity of P-glycoprotein (Chambers et al., 1990, 1993; Hamada et al., 1987; Ma et al., 1991; Yu et al., 1991). Therefore, we investigated the effect of protein

kinase C inhibitors (H-7, staurosporine, Ro31-8220, K252a and protein kinase C inhibitor peptide (19–31)) or an activator (PMA) on the uptake of [^{3}H]vincristine. Uptake was increased by protein kinase C inhibitors, but decreased by PMA (Fig. 5), suggesting that protein kinase C activity is also an important determinant of the functional activity of P-glycoprotein in MBEC4 cells. We examined the change in the phosphorylation of P-glycoprotein in the presence of H-7 or PMA to directly clarify the relationship between the functional activity of P-glycoprotein and the phosphorylation of P-glycoprotein. The phosphorylation of P-glycoprotein was increased by PMA, whereas it was reduced in the presence of H-7 (Fig. 6B). The phosphorylation of P-glycoprotein was enhanced in the presence of 5 μM quercetin (Fig. 6A). These findings indicate that there is a close relationship between the enhancement of the phosphorylation of P-glycoprotein by quercetin and the transport of [^{3}H]vincristine. As shown in Fig. 6, there was no effect on the phosphorylation of P-glycoprotein in the presence of 25 and 50 μM quercetin. There are two possible explanations for these phenomena. One is the inactivation of protein kinase C by high concentrations (25 and 50 μM) of quercetin. It has been reported that protein kinase C is activated by acute treatment with PMA, an activator of protein kinase C, while it is inactivated by long-term treatment with PMA (Ahn et al., 1996). Therefore, it is plausible that a high concentration of quercetin may inactivate protein kinase C in a biphasic manner. The other possibility is that a phosphatase, a dephosphorylation enzyme, is activated in the presence of a high concentration of quercetin. There was no effect of quercetin on the expression of P-glycoprotein measured by ELISA (data not shown). Although 50 μM quercetin significantly decreased P-glycoprotein expression, this finding could not fully explain the increase in [^{3}H]vincristine uptake. Therefore, a high concentration of quercetin may inhibit the function of P-glycoprotein. However, it is unclear whether the decrease in the P-glycoprotein-mediated efflux of [^{3}H]vincristine is due to altered phosphorylation of P-glycoprotein or due to the direct inhibition of P-glycoprotein by the inhibitors.

The K_p value of [^{3}H]vincristine was significantly decreased by the co-administration of 0.1 mg/kg of quercetin, but it was increased by 1.0 mg/kg of quercetin (Table 1). It is likely that the increased K_p value of [^{3}H]vincristine was due to the inhibition of P-glycoprotein function and/or alteration of the unbound fraction of [^{3}H]vincristine in plasma by the co-administration of 1.0 mg/kg quercetin. However, the unbound fraction of [^{3}H]vincristine in plasma was large (0.4), and the protein binding of [^{3}H]vincristine was constant at concentrations lower than 10 μM (31). Furthermore, quercetin mainly bind to albumin (Boulton et al., 1998), while the binding of vincristine to albumin is quite low (10%) (Donigian and Owellen, 1973). Therefore, the alteration of the K_p value by the co-administration of quercetin may not be due to a change in the unbound fraction of vincristine in plasma. The biphasic effect of quercetin on the uptake of [^{3}H]vincristine was also seen in the brain-tissue distribution study in vivo. To investigate whether the concentration of quercetin in the in vivo experiment corresponded to that in the in vitro study, we determined the plasma concentration of quercetin after intravenous administration of quercetin. The plasma concentration of quercetin at 30 s to 30 min after intravenous administration of 0.1 mg/kg or 1.0 mg/kg quercetin ranged from 0.006 ± 0.0016 to 0.405 ± 0.014 μM or 0.017 ± 0.008 to 4.57 ± 0.82 μM, respectively. Thus, the plasma concentration of quercetin at 30 s was approximately 10 times lower than the concentration in the in vitro experiments. It is not clear what caused this difference. One of the possibilities is a difference in affinity for quercetin and/or vincristine among the *mdr* isoforms. *Mdr1a* mRNA is mainly expressed in mouse brain in vivo, while only *mdr1b* mRNA is found in MBEC4 cells in vitro. Indeed, it has been reported that (1) *mdr1a* P-glycoprotein is a more efficient efflux pump than *mdr1b* P-glycoprotein, (2) a single class of vinblastine binding sites is present in *mdr1b* P-glycoprotein-expressing cells, whereas there appear to be two classes of such sites in *mdr1a* P-glycoprotein-expressing cells, and (3) progesterone inhibits vinblastine efflux and increases drug sensitivity to vinblastine with more potency in *mdr1b* P-glycoprotein-expressing cells than in *mdr1a* P-glycoprotein-expressing cells (Yang et al., 1990).

Since P-glycoprotein is distributed not only in brain capillary endothelial cells, which form the blood–brain barrier, but also in the liver, kidney and other normal tissues (Thiebau et al., 1987; Bradley et al., 1990), care may be needed to avoid the consumption of meals containing bioflavonoids concomitantly with the administration of drugs that are substrates of P-glycoprotein, since the drug-bioflavonoid interaction may change drug pharmacokinetics and give rise to unexpected side effects.

In conclusion, we found a biphasic, concentration-dependent effect of quercetin on the blood–brain barrier transport of [^{3}H]vincristine. Efflux was indirectly activated as a result of an increased phosphorylation of P-glycoprotein at a low concentration of quercetin, while it was inhibited because of inhibition of P-glycoprotein at a high concentration of quercetin.

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