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Research Paper

St. John's Wort Constituents Modulate P-glycoprotein Transport Activity at the Blood-Brain Barrier

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Purpose. The purpose of this study was to investigate the short-term signaling effects of St. John's Wort (SJW) extract and selected SJW constituents on the blood-brain barrier transporter P-glycoprotein and to describe the role of PKC in the signaling.
Methods. Cultured porcine brain capillary endothelial cells (PBCEC) and freshly isolated brain capillaries from pig were used as *in vitro*/*ex vivo* blood-brain barrier model. SJW modulation of P-glycoprotein function was studied in PBCEC using a calcein-AM uptake assay and in isolated pig brain capillaries using the fluorescent cyclosporine A derivative NBD-CSA and confocal microscopy.
Results. SJW extract and the constituents hyperforin, hypericin, and quercetin decreased P-glycoprotein transport activity in a dose- and time-dependent manner. SJW extract and hyperforin directly inhibited P-glycoprotein activity, whereas hypericin and quercetin modulated transporter function through a mechanism involving protein kinase C. Quercetin at high concentrations decreased P-glycoprotein transport activity, but increased transporter function at low concentrations. This increase in P-glycoprotein activity was likely due to trafficking and membrane insertion of vesicles containing transporter protein.
Conclusions. Our findings provide new insights into the short-term interaction of SJW with P-glycoprotein at the blood-brain barrier. They are of potential relevance given the wide use of SJW as OTC medication and the importance P-glycoprotein has for CNS therapy.

KEY WORDS: blood-brain barrier; P-glycoprotein; protein kinase C; regulation; St. John's wort.

INTRODUCTION

St. John's Wort (SJW, Hypericum perforatum) is one of the best-described herbal medications that has been used for over 2000 years. The spectrum of SJW indications is remarkably wide and includes gastrointestinal disorders, skin disease, and mucosal lesions. For the most part, however, SJW is used to treat conditions such as mild depression, anxiety, restlessness, and sleeping disorders (1,2). For these indications, SJW is a popular prescription and over-the-counter herbal drug that is used by millions of people worldwide. In the US alone, total annual sales account for about $140 million (3,4).

SJW extracts are complex mixtures of more than 150 compounds. These include phloroglucinols such as hyperforin and adhyperforin, naphtodianthrones such as hypericin and pseudohypericin, flavonoids such as quercetin, quercitrin and rutin, as well as other constituents like proanthocyanidines or biflavones (2). The therapeutic effect of SJW is considered to be based on multiple compounds, among which the hyperforins, hypericins and several flavonoids are the most important. However, the exact pharmacological mechanism of SJW and its constituents is still unclear.

Although SJW has been shown to be effective in mild depression and well tolerated when given alone, there is ample evidence for drug-drug interactions causing severe clinical complications when SJW is co-administered with other drugs. For example, transplant rejections have been reported in patients taking SJW alongside the immunosuppressant cyclosporine A (5,6). Other drugs affected by SJW include statins, oral contraceptives, anticoagulants, HIV-protease inhibitors, chemotherapeutics or cardiovascular drugs (7,8). All of these drugs are eliminated through metabolism by the phase-I enzyme, CYP3A4, and through excretion by ABC efflux transporters. Since SJW affects both CYP450 enzymes and efflux transporters, it changes the pharmacokinetics of a large number of therapeutics resulting in drug-drug interactions and side effects. This phenomenon

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ABBREVIATIONS: HC, Hypericin; HF, Hyperforin; PBCEC, Porcine brain capillary endothelial cells; PKC, Protein kinase C; PMA, Phorbol 12-myristate 13-acetate; PXR, Pregnane X receptor; QE, Quercetin; SJW, St. John's wort.

has recently been linked to the xenobiotic-activated nuclear transcription factor, pregnane X receptor, PXR (*NR1I2*) (9). In this regard, we recently conducted a study where we demonstrated that hyperforin-mediated activation of PXR leads to up-regulation of P-glycoprotein, the most prominent drug efflux transporter at the blood-brain barrier (10). This was an interesting finding, given the importance of P-glycoprotein for protecting the brain and limiting entry into the CNS of a large number of therapeutic drugs.

In contrast to SJW-induced longer-term effects on P-glycoprotein through PXR, several other reports describe direct interactions of SJW constituents with P-glycoprotein over the short-term (11–14). While most of these studies focused on the intestine, little is known about such interactions at the blood-brain barrier. Given that hypericin and quercetin are both known to modulate PKC (15,16), one mechanism by which SJW constituents could rapidly affect P-glycoprotein is signaling through protein kinase C (PKC). Using rat brain capillaries, Hartz *et al.* recently showed that short-term exposure (1 h) with a PKC activator resulted in a concentration-dependent decrease of P-glycoprotein transport function (17). Bates *et al.* demonstrated in a MDR1-overexpressing cell line that activation of PKC enhanced P-glycoprotein phosphorylation and transport function, while treatment with PKC inhibitors decreased phosphorylation and drug transport function in MDR cells within 1 h (18). A signaling pathway that is unrelated to P-glycoprotein phosphorylation but involves PKC inhibition was found by Castro *et al.* in a mouse fibroblast cell line (19). So far, nothing is known about interactions of SJW with P-glycoprotein involving PKC signaling. Since P-glycoprotein is critical for active and selective barrier function and treatment of CNS disorders and since SJW is a widely used herbal over-the-counter drug, this topic is of particular relevance.

In the present study we therefore focused on SJW signaling changes in blood-brain-barrier P-glycoprotein transport function over the short-term and aimed at identifying the role of PKC in this signaling. Our data provide important new insights into the interaction of SJW constituents with P-glycoprotein at the blood-brain barrier and are of clinical relevance given the importance of this transporter for CNS therapy.

MATERIALS AND METHODS

Materials

Fluorescent NBD-CSA (NBD-cyclosporin A) was provided by Dr. R. Wenger, Basel, Switzerland (20). PSC833 (3′-oxo-4-butenyl-4-methyl-threonine(1), (valine(2)) cyclosporin) was a kind gift from Novartis (Basel, CH). Hypericum perforatum compounds were provided by Prof. Dr. Schubert-Zsilavecz, Frankfurt, Germany (Hypericum extract) and Dr. Wilmar Schwabe, Karlsruhe, Germany (pure substances). Calcein-AM and FITC-conjugated rabbit anti-mouse IgG were purchased from Invitrogen, Karlsruhe, Germany. Phorbol-12-myristate-13-acetate (PMA) and bisindolylmaleimide I (BIM) were obtained from Calbiochem, Darmstadt, Germany. 4α-phorbol 12-myristate 13-acetate (4α-PMA) was purchased from Promega, Mannheim, Germany. Brefeldin A was purchased from Sigma-Aldrich Chemie, Munich, Germany. Mab C219 was obtained from Alexis, Grünberg, Germany. All cell culture supplies were obtained from Biochrom, Berlin, Germany. Dispase II, collagenase-dispase and rat tail collagen were from Roche Diagnostics, Mannheim, Germany. All other chemicals were obtained from commercial sources at the highest purity available.

Isolation of Porcine Brain Capillaries

Brain capillaries from pig were isolated as described previously (21). All steps were carried out using ice-cold artificial cerebrospinal fluid (aCSF) (103 mM NaCl, 4.7 mM KCl, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4 \times 7H_2O$, 25 mM $NaHCO_3$, 2.5 mM $CaCl_2$, 10 mM D-glucose, 10 mM HEPES, 1 mM sodium pyruvate, 100 μg/ml penicillin/streptomycin, 100 μg/ml gentamycin, pH 7.4). Brains were cleaned from meninges, choroid plexus and large superficial blood vessels. Cortical grey matter was collected, minced with a scalpel and homogenized in aCSF using a Potter-Elvejhem tissue grinder. After adding dextran solution (15% final concentration), brain capillaries were separated from the homogenate by density centrifugation (5,800 g, 10 min, 4°C), resuspended in aCSF containing 0.5% BSA, and filtered through a 150 μm Polymon® mesh. The filtrate was passed over a glass bead column (Ø 0.45–0.50 mm), and capillaries adhering to the beads were collected by gentle agitation. Capillaries were washed, resuspended in pregassed aCSF, and immediately used for transport experiments.

Isolation of Endothelial Cells and Primary Cell Culture

Porcine brain capillary endothelial cells (PBCEC) were isolated following recently described protocols (22). Cortical grey matter was obtained as described above and collected in preparation medium (Earle's Medium 199 supplemented with 0.8 mM L-glutamine, 100 μg/ml penicillin/streptomycin, 100 μg/ml gentamicin and 10 mM HEPES, pH 7.4). Dispase II was added (final concentration of 0.5%), and brain tissue was digested at 37°C for 2 h. After centrifugation (1,000 g, 10 min, 4°C), floating supernatant and myelin were discarded, and the capillary pellet was resuspended in preparation medium containing 15% dextran. Capillaries were separated from remaining tissue by centrifugation (5,800 g, 15 min, 4°C) and subsequently digested with 0.1% collagenase/dispase in preparation medium at 37°C for 1.5 h. The resulting cell suspension was filtered through a 150 μm Polymon® mesh, centrifuged (130 g, 10 min, 4°C) and added to a discontinuous Percoll® gradient (1.03 g/ml and 1.07 g/ml). After centrifugation (1,250 g, 10 min, 4°C), endothelial cells enriched at the interface between the 2 Percoll® solutions were collected, washed and filtered through a 150 μm Polymon® mesh. The final cell suspension was seeded at 250,000 cells/cm^2 onto collagen-coated 8-well chamber slides or 96-well plates. Cells were cultured under standard cell culture conditions using preparation medium without gentamicin but with 10% horse serum. One day prior to experiments, the medium was changed to DMEM/Ham's F12 (1:1) supplemented with 2 mM L-glutamine, 100 μg/ml penicillin/streptomycin and 10 mM HEPES. Confluent cell monolayers were used after 7 days in culture.

Immunochemistry

Confluent PBCEC, grown on chamber slides, were washed with phosphate-buffered saline (PBS) and fixed with 4% paraformaldehyde in PBS for 30 min. After washing, cells were permeabilized with 0.1% Triton-X-100 in PBS for 30 min. Non-specific binding was prevented by incubation with 5% rabbit serum in PBS for 30 min. Subsequently, cells were washed and incubated overnight at 4°C with monoclonal C219 antibody (1:20) against P-glycoprotein. Cells were washed again and incubated for 60 min at 37°C with FITC-labeled secondary IgG antibody (1:20) and 10 μg/ml propidium iodide. Finally, cells were washed, embedded with Aqua Poly Mount®, and sealed using a coverslip and commercially available colorless nail varnish.

Isolated porcine brain capillaries were transferred onto slides and fixed for 20 min with 3% paraformaldehyde/0.1% glutaraldehyde/3.4% sucrose in PBS. After washing, capillaries were permeabilized for 15 min with 1% Triton X-100. Capillaries were blocked for 30 min with 2% BSA in PBS, washed again, and subsequently incubated for 1 h at room temperature with C219 antibody (1:20). After washing, capillaries were incubated for 40 min at 37°C with FITC-conjugated secondary IgG antibody (1:50) and 10 μg/ml propidium iodide. Immunostained P-glycoprotein in PBCEC and brain capillaries was visualized by confocal microscopy (Leica DM IRBE laser scanning microscope, Bensheim, Germany) using the 488 nm laser line and a 40X oil immersion objective (NA 1.2).

Membrane Isolation and Western Blotting

PBCEC and isolated brain capillaries were homogenized in CellLytic™ MT mammalian tissue lysis buffer (Sigma-Aldrich Chemie, Munich, Germany) containing protease inhibitor cocktail (Roche Diagnostics GmbH, Mannheim, Germany) using a glass homogenizer. Cell and tissue lysates were first centrifuged at 10,000 g for 30 min at 4°C. Supernatants were then centrifuged at 100,000 g for 90 min at 4°C; resulting crude plasma membranes were resuspended in lysis buffer. Protein concentrations were determined using BioRad Protein Assay. Membrane proteins were separated on 7.5% SDS-polyacrylamide gels and blotted on Immuno-Blot PVDF membranes. Membranes were blocked for 1 h with 1% BSA/1% milk powder/0.5% Tween in PBS and incubated overnight at 4°C with monoclonal C219 antibody (1:100). After washing, membranes were incubated for 1 h with HRP-conjugated secondary IgG (1:1000). Protein bands were detected using Western Lightning™ Chemiluminescence Reagent (PerkinElmer, Rodgau, Germany) and visualized with a ChemiDoc XRS system (BioRad, Munich, Germany).

NBD-CSA Transport in Capillaries

Fluorescence microscopy with isolated capillaries was done according to Miller *et al.* (23). Preliminary experiments revealed that NBD-CSA transport was about 25% higher at room temperature than at 37°C. Therefore, all further experiments were conducted at room temperature. In brief, freshly isolated capillaries were transferred to teflon incubation chambers, preincubated for 30 min with test compounds (with or without corresponding PKC modulators) in aCSF, followed by incubation with 2 μM NBD-CSA for an additional 30 min. Confocal microscopy was performed with a Leica DM IRBE laser scanning microscope (Leica, Bensheim, Germany) using the 488 nm laser line. Capillaries were imaged using a 63X or 40X water immersion objective (NA 1.2). For each treatment, confocal images of 5–8 capillaries were acquired. Fluorescence intensities were measured from stored images using Scion Image software (Scion Corp., Frederik, MD) as described previously (23).

Calcein-AM Uptake in PBCEC

Confluent PBCEC monolayers were washed 3 times with 37°C Krebs-Ringer buffer (142 mM NaCl, 3 mM KCl, 1.5 mM K_2HPO_4, 1.2 mM $MgCl_2$, 1.4 mM $CaCl_2$, 4 mM Glucose, 10 mM HEPES) and incubated with increasing concentrations of test compound for 15 min at 37°C. For protein kinase C experiments, cells were incubated for 30 min with the indicated test compounds with or without PKC modulators. Subsequently, Calcein-AM was added to a final concentration of 1 μM for 30 min at 37°C. PBCEC were washed 3 times with ice-cold KRB and lysed with 1% Triton-X-100. Fluorescence was measured using a Fluoroskan Ascent® plate reader (Thermo Electron Corporation, Dreieich, Germany). Intracellular fluorescence was obtained by subtracting background fluorescence of control wells and calcein uptake was expressed as % of control. For each compound, at least three independent experiments were performed with cells from different cell preparations.

Cytotoxicity Assay

All tested compounds were prepared as stock solutions in DMSO or methanol. Like in all other experiments, the final solvent concentration on the cells did not exceed 1%. Seven-day-old confluent PBCEC in 96-well plates were exposed to the test compounds for 2 h. After exposure, AlamarBlue® stock solution (Serotec, Düsseldorf, Germany) was added for 6 h at 37°C under 5% CO_2 atmosphere. The AlamarBlue® viability test utilizes a fluorescent dye to detect metabolic activity, and in this assay, fluorescence correlates with cell viability. Fluorescence was measured using a Fluoroskan Ascent® plate reader (Thermo Electron Corporation, Dreieich, Germany) and was expressed as % of control. Drugs were only used at concentrations that did not have an effect on cell viability as determined by the AlamarBlue® test.

Statistics

Data are given as mean ± S.E.M. Means were judged to be significantly different when $p<0.05$ using an unpaired Student's *t*-test.

RESULTS

P-glycoprotein Expression in Porcine Brain Capillary Endothelial Cells and Brain Capillaries

We detected P-glycoprotein expression in cultured PBCEC and isolated capillaries by immunostaining and

Western blotting using C219 antibody (Fig. 1). Fig. 1A shows immunolabeled P-glycoprotein (green) in spindle-shaped brain capillary endothelial cells; nuclei were counter-stained with propidium iodide (red). There was no signal for P-glycoprotein in cells that were incubated with secondary antibody alone (Fig. 1B). In isolated brain capillaries from pig, we observed that immunoreactive P-glycoprotein was localized to the capillary membrane (Fig. 1C); negative controls showed no P-glycoprotein immunofluorescence (Fig. 1D). Western blot analysis of PBCEC and capillary crude membranes (Fig. 1E and F) showed one distinct band at 170 kDa indicating P-glycoprotein expression in both tissues. We did not detect a signal for P-glycoprotein in whole brain lysate, suggesting transporter enrichment in the plasma membrane; liver homogenate was used as positive control. These findings indicate that P-glycoprotein is expressed in the brain capillary endothelium in pig, which is in agreement with previous findings in rat and mouse (24,25).

P-glycoprotein Transport Activity in Porcine Capillary Endothelial Cells and Brain Capillaries

To study P-glycoprotein transport activity in PBCEC, we used a previously developed assay that is based on the intracellular accumulation of fluorescent calcein (22). In this assay, cells are first exposed to the non-fluorescent P-glycoprotein substrate, calcein-acetoxymethylester (calcein-AM). Calcein-AM is highly lipophilic and, therefore, easily penetrates into cells, where it is rapidly cleaved to fluorescent calcein. Since calcein-AM is a P-glycoprotein substrate, inhibition of transporter-mediated calcein-AM efflux increases intracellular calcein fluorescence. Thus, an increase in intracellular calcein fluorescence is a measure of P-glycoprotein transport activity. In this assay, the specific P-glycoprotein inhibitor, PSC833 (positive control for maximal P-glycoprotein inhibition), increased intracellular calcein fluorescence in a concentration-dependent manner (Fig. 2A). Since PBCEC also express multidrug resistance proteins (MRPs) and since calcein-AM is a Mrp1 substrate and calcein is a substrate for both Mrp1 and Mrp2 (26–28), we tested this assay on its specificity for P-glycoprotein. Fig. 2B shows that the Mrp inhibitors, MK571 and LTC4, did not affect intracellular calcein fluorescence. In addition, fumitremorgin C, an inhibitor for the breast cancer resistance protein, BCRP, did not have any effect on calcein fluorescence below 10 μM (data not shown). Thus, in PBCEC, the calcein assay is specific for assessing P-glycoprotein transport activity.

We also determined P-glycoprotein activity with our recently established assay that utilizes freshly isolated, intact brain capillaries *ex vivo* (23). In this assay, isolated capillaries are exposed to the fluorescent, P-glycoprotein-specific substrate, NBD-CSA (NBD-cyclosporin A) and monitored by confocal microscopy. NBD-CSA accumulation in the capillary lumen is then quantitated by digital image analysis (23). Note that in this assay, an increase in luminal NBD-CSA fluorescence corresponds to an increase in P-glycoprotein transport activity, whereas a decrease in luminal fluorescence corresponds to a decrease in transporter functional activity. Accordingly, exposing isolated pig brain capillaries to 2 μM NBD-CSA resulted in an increase in capillary luminal fluorescence over time (Fig. 2C). Accumulation of NBD-CSA in capillary lumens was saturable and reached steady state within approximately 30 min. Fig. 2D shows a concentration-dependent decrease of luminal NBD-CSA fluorescence when brain capillaries from pig were exposed to PSC833; a maximal effect was observed with 5 μM PSC833. Luminal NBD-CSA accumulation was also decreased by ivermectin, cyclosporin A, and the metabolic inhibitor, NaCN (data not shown) indicating an active, P-glycoprotein-mediated process. In agreement with our previous reports for rat (23,29), the Mrp inhibitors, MK571 and LTC_4, did not affect NBD-CSA

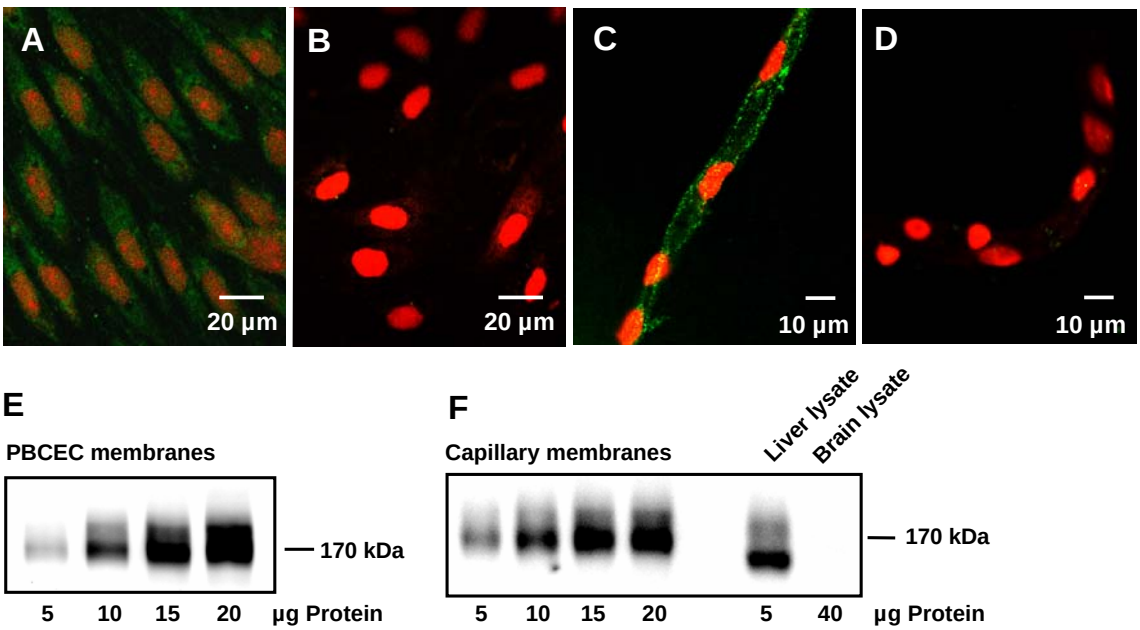

Fig. 1. P-glycoprotein expression in porcine brain capillary endothelial cells and brain capillaries. Representative images showing P-glycoprotein immunostaining (*green*) in PBCEC (**A**) and isolated pig brain capillaries (**C**); cell nuclei were counterstained with propidium iodide (*red*). Control samples (**B** and **D**) were processed without primary antibody against P-glycoprotein. Western Blot analysis of P-glycoprotein in isolated membranes of PBCEC (**E**) and pig brain capillaries (**F**).

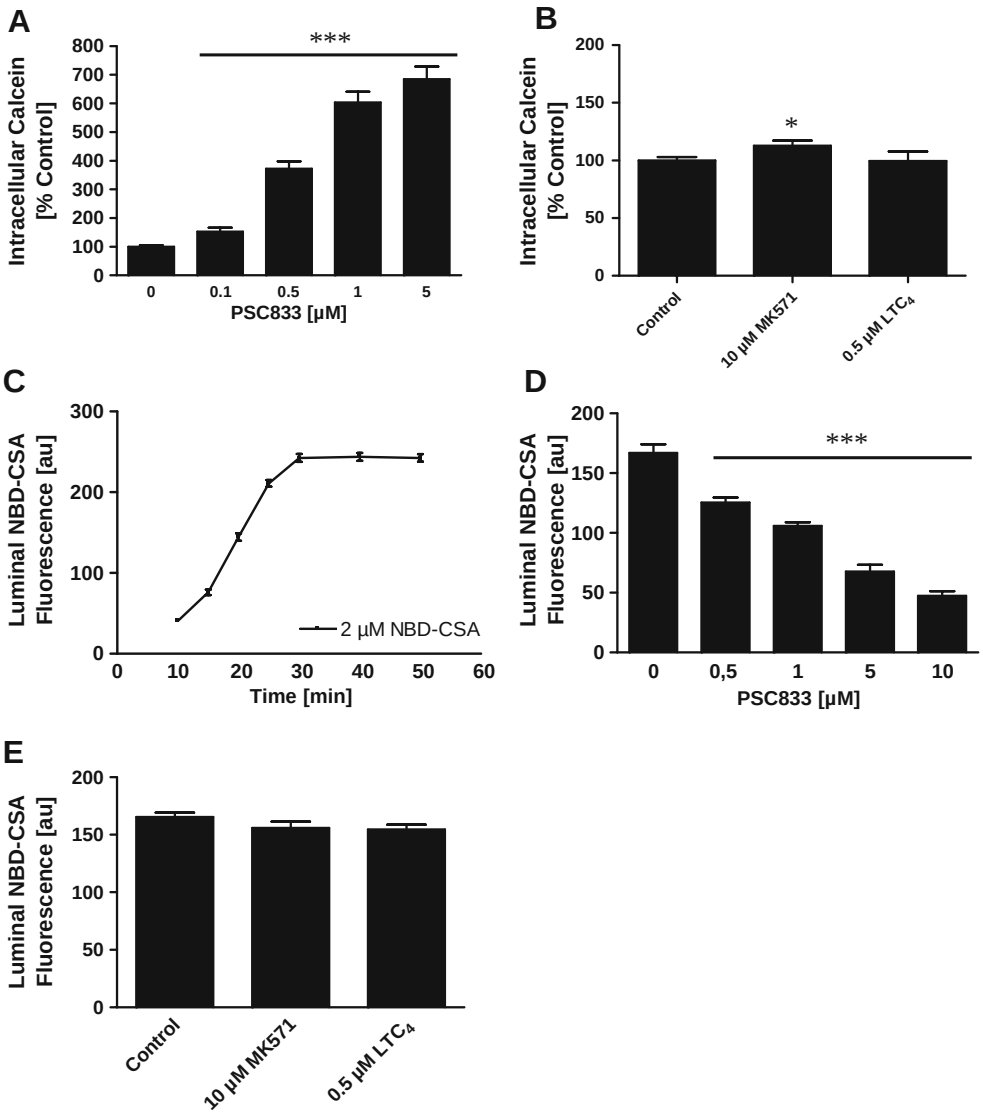

Fig. 2. P-glycoprotein transport activity in porcine capillary endothelial cells and brain capillaries. **A** Dose response of the P-glycoprotein-specific inhibitor, PSC833, on intracellular calcein accumulation in PBCEC. **B** Mrp inhibitors did not affect intracellular calcein fluorescence. **C** Time course of NBD-CSA accumulation in lumens of isolated pig brain capillaries. **D** PSC833 dose response on luminal NBD-CSA accumulation in pig brain capillaries. **E** Mrp inhibitors did not affect luminal NBD-CSA fluorescence. Data for calcein assay (**A** and **B**) are means ± SEM ($n=6$). Data for luminal NBD-CSA fluorescence (**C**–**E**) are means ± SEM for 5–8 capillaries from a single preparation (pooled tissue from 5 to 6 pig brains). Units are arbitrary fluorescence units (0–255). Statistics: *$p<0.05$, ***$p<0.001$, significantly different from controls (Student's *t*-test).

luminal fluorescence, indicating that this assay is also P-glycoprotein-specific in pig (Fig. 2E).

Together, using two assays to measure P-glycoprotein transport activity in cultured PBCEC monolayers *in vitro* and isolated brain capillaries *ex vivo*, we demonstrate that P-glycoprotein was functionally active at the pig blood-brain barrier and that transport was P-glycoprotein-specific, concentrative, and active.

St. John's Wort Effect on P-glycoprotein Transport Activity in PBCEC and Brain Capillaries

In PBCEC monolayers, 45 min exposure to SJW extract increased intracellular calcein fluorescence in a concentration-dependent manner. At the lowest concentration of 100 ng/ml, SJW extract increased calcein fluorescence in PBCEC to 130±10% of controls (100%). At the highest concentration of 5 µg/ml SJW extract, intracellular calcein fluorescence increased to 314±23% of controls (Fig. 3A). Similarly, hyperforin increased intracellular calcein to a maximum of 340±32% (Fig. 3B). Hypericin at the highest concentration of 50 µM had only a weak effect on intracellular calcein fluorescence, which was increased to 161±15% (Fig. 3C). As shown in Fig. 3D, exposing PBCEC to quercetin resulted in a concentration-dependent biphasic effect. At lower quercetin concentrations (1–10 µM), we observed a significant decrease of intracellular calcein fluorescence below control values (1 µM: 73%; 5 µM: 80%;

10 μM: 86%), suggesting activation of P-glycoprotein transport activity. At concentrations above 10 μM quercetin, however, this phenomenon disappeared, and intracellular calcein fluorescence increased, suggesting P-glycoprotein inhibition. Note that at the concentrations used in our experiments, none of these compounds had any effect on PBCEC viability in the AlamarBlue® cytotoxicity assay (data not shown).

P-glycoprotein transport activity was also reduced in isolated pig brain capillaries that were exposed to SJW extract, hyperforin, hypericin, and quercetin. Fig. 4A shows a control capillary that was incubated with 2 μM NBD-CSA for 30 min. Note that under our imaging conditions, there was no fluorescence in the bath containing NBD-CSA, but fluorescence accumulated in the capillary lumen, indicating concentrative transport. In the presence of 5 μg/ml SJW extract, accumulation of NBD-CSA in capillary lumens was significantly reduced (Fig. 4B). Digital image analysis revealed that 5 μg/ml SJW extract decreased luminal NBD-CSA fluorescence to about 40% of controls (Fig. 4C). Hyperforin and hypericin alone also decreased luminal NBD-CSA fluorescence in a concentration-dependent manner. Fig. 4D and E show that 10 μM hyperforin reduced luminal NBD-CSA fluorescence to about 40% of controls, and 10 μM hypericin reduced luminal fluorescence to 45% of control values. Quercetin displayed a concentration-dependent biphasic effect also in brain capillaries (Fig. 4F). Luminal NBD-CSA fluorescence increased up to 38% of controls with 1 μM quercetin, suggesting P-glycoprotein activation. Quercetin concentrations above 10 μM, however, decreased NBD-CSA fluorescence in capillary lumens.

In addition, we used PSC833 as a positive control for maximal P-glycoprotein inhibition in all experiments. With 5 μM PSC833, calcein fluorescence in PBCEC averaged 633% of controls (100%), and NBD-CSA fluorescence in capillary lumens averaged 32% of controls (100%). Thus, SJW, hyperforin, hypericin, and quercetin were significantly less potent than PSC833 in modulating P-glycoprotein transport activity.

PKC is Involved in SJW Modulation of P-glycoprotein Transport Activity

The SJW constituents hypericin and quercetin are known to modulate protein kinase C (PKC) (15,16). In addition, PKC has previously been reported to be involved in P-glycoprotein regulation (17). To determine PKC involvement in the modulation of P-glycoprotein transport activity by SJW and its constituents, we first tested the effect of PKC modulation on P-glycoprotein transport activity and exposed PBCEC and isolated pig brain capillaries to the PKC activator, phorbol 12-myristate 13-actetate (PMA), and the PKC inhibitor, bis-indolylmaleimide I (BIM). We performed dose response and time course experiments to determine PMA and BIM concentrations and time points at which P-glycoprotein activity was maximally affected by these compounds (see Supplementary Material) and used this information for our next experiments. Fig. 5A shows that 30 min preincubation of PBCEC with 10 nM PMA decreased intracellular calcein fluorescence, suggesting that P-glycoprotein transport activity was stimulated. There was no effect with the inactive PMA analogue, 4α-PMA, which we used as a

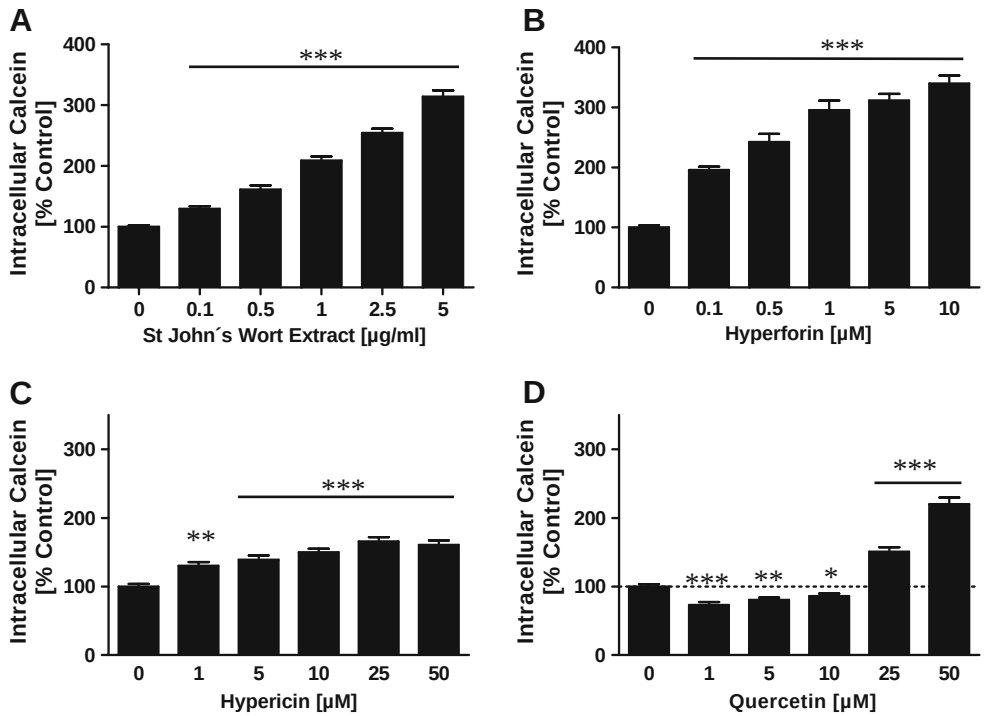

Fig. 3. St. John's Wort effect on P-glycoprotein transport activity in PBCEC. Effect of St. John's Wort extract (**A**), hyperforin (**B**), hypericin (**C**), and quercetin (**D**) on intracellular calcein accumulation in PBCEC. Data are means ± SEM (n=6). Statistics: *p<0.05; **p<0.01; ***p<0.001, significantly different from controls (Student's t-test).

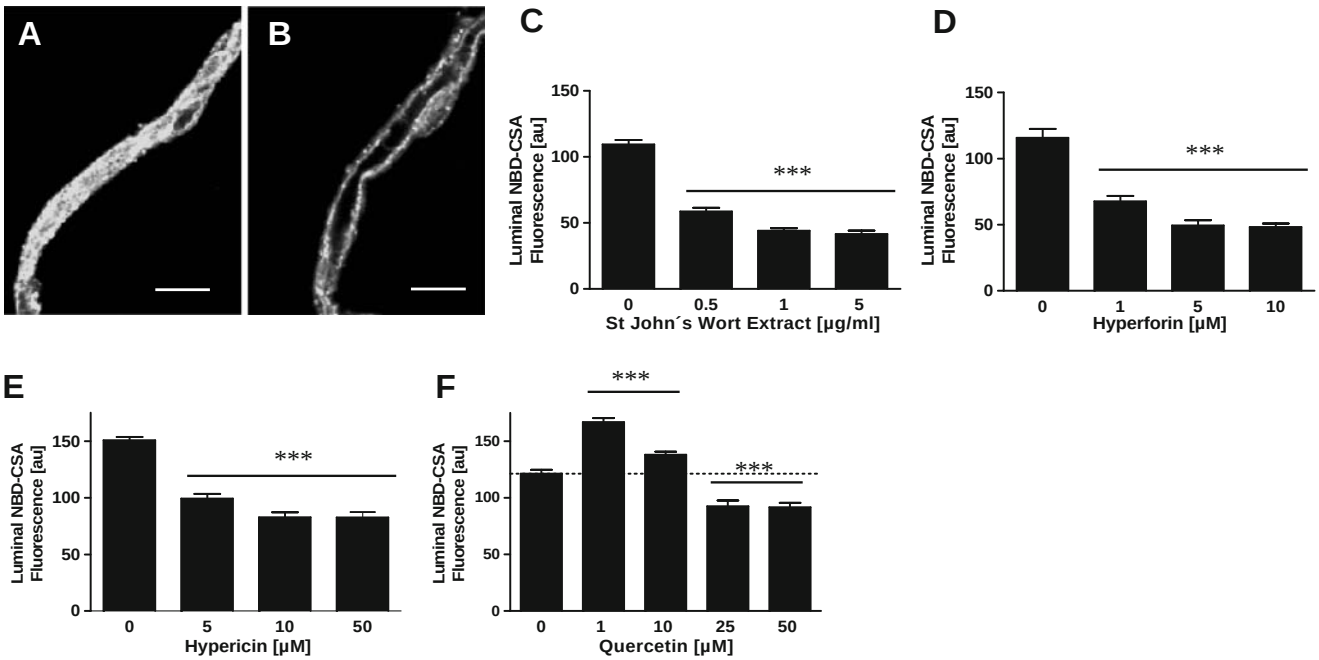

Fig. 4. St. John's wort effect on P-glycoprotein transport activity in brain capillaries. (**A**) Representative image of control capillary exposed to 2 µM NBD-CSA. Note the luminal accumulation of NBD-CSA indicated by high fluorescence. (**B**) SJW extract abolished luminal NBD-CSA accumulation. Effect of St. John's wort extract (**C**), hyperforin (**D**), hypericin (**E**), and quercetin (**F**) on luminal NBD-CSA accumulation in isolated pig brain capillaries. Data are means ± SEM for 5–8 capillaries from a single preparation (pooled tissue from 5 to 6 pig brains). Units are arbitrary fluorescence units (0–255). Statistics: $*p<0.05$; $**p<0.01$; $***p<0.001$, significantly different from controls (Student's *t*-test).

negative control for PKC activation. In contrast, the PKC inhibitor BIM significantly increased intracellular calcein fluorescence, suggesting PKC inhibition reduced P-glycoprotein transport function. Exposing PBCEC to both PMA and BIM had no effect on calcein fluorescence, suggesting BIM abolished PMA-mediated activation of P-glycoprotein transport activity. These observations were consistent with results obtained from experiments in isolated pig brain capillaries *ex vivo* (Fig. 5B). PKC activation with PMA increased NBD-CSA fluorescence in capillary lumens, suggesting activation of P-glycoprotein activity. The inactive PMA analogue 4α-PMA had no effect, but BIM, which is used to block PKC, decreased luminal NBD-CSA fluorescence, suggesting reduced P-glycoprotein function. These findings strongly suggest that PKC is implicated in SJW modulation of P-glycoprotein at the pig blood-brain barrier and that PKC is one regulatory protein that modulates blood-brain barrier P-glycoprotein function in pig.

To connect PKC signaling to P-glycoprotein with the SJW effect on the transporter, we exposed PBCEC and isolated

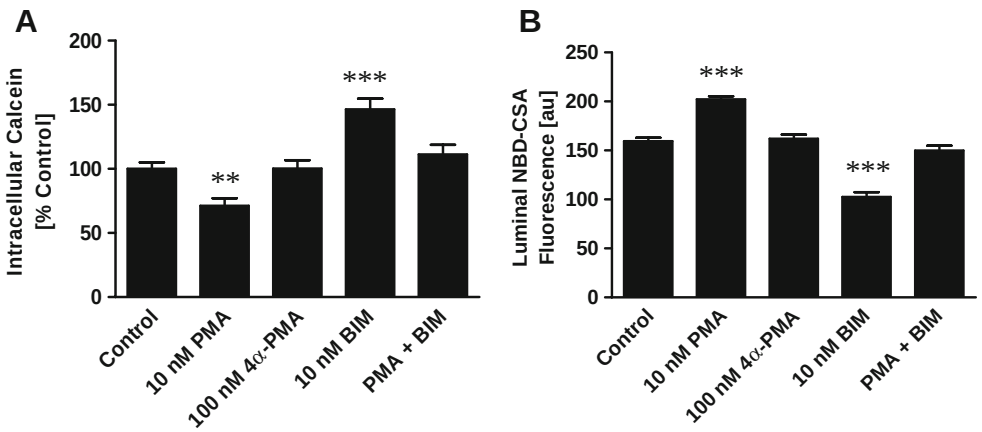

Fig. 5. PKC modulators affect P-glycoprotein transport activity. PKC activation with PMA and PKC inhibition with BIM affect intracellular calcein fluorescence in PBCEC (**A**) and luminal NBD-CSA fluorescence in isolated pig brain capillaries. Data for calcein assays (**A**) are means ± SEM ($n=6$). Data for luminal NBD-CSA fluorescence (**B**) are means ± SEM for 5–8 capillaries from a single preparation (pooled tissue from 5 to 6 pig brains). Units are arbitrary fluorescence units (0–255). Statistics: $**p<0.01$; $***p<0.001$, significantly different from controls (Student's *t*-test).

brain capillaries to SJW extract and its constituents with and without the PKC modulators PMA and BIM. In PBCEC, PMA partly counteracted the SJW-induced increase of intracellular calcein fluorescence, but not the hyperforin-induced effect (Fig. 6A and B). In contrast, PMA completely abolished the hypericin- and quercetin-mediated increase in calcein fluorescence (Fig. 6C and D), suggesting both compounds reduced P-glycoprotein transport activity through PKC inhibition. In

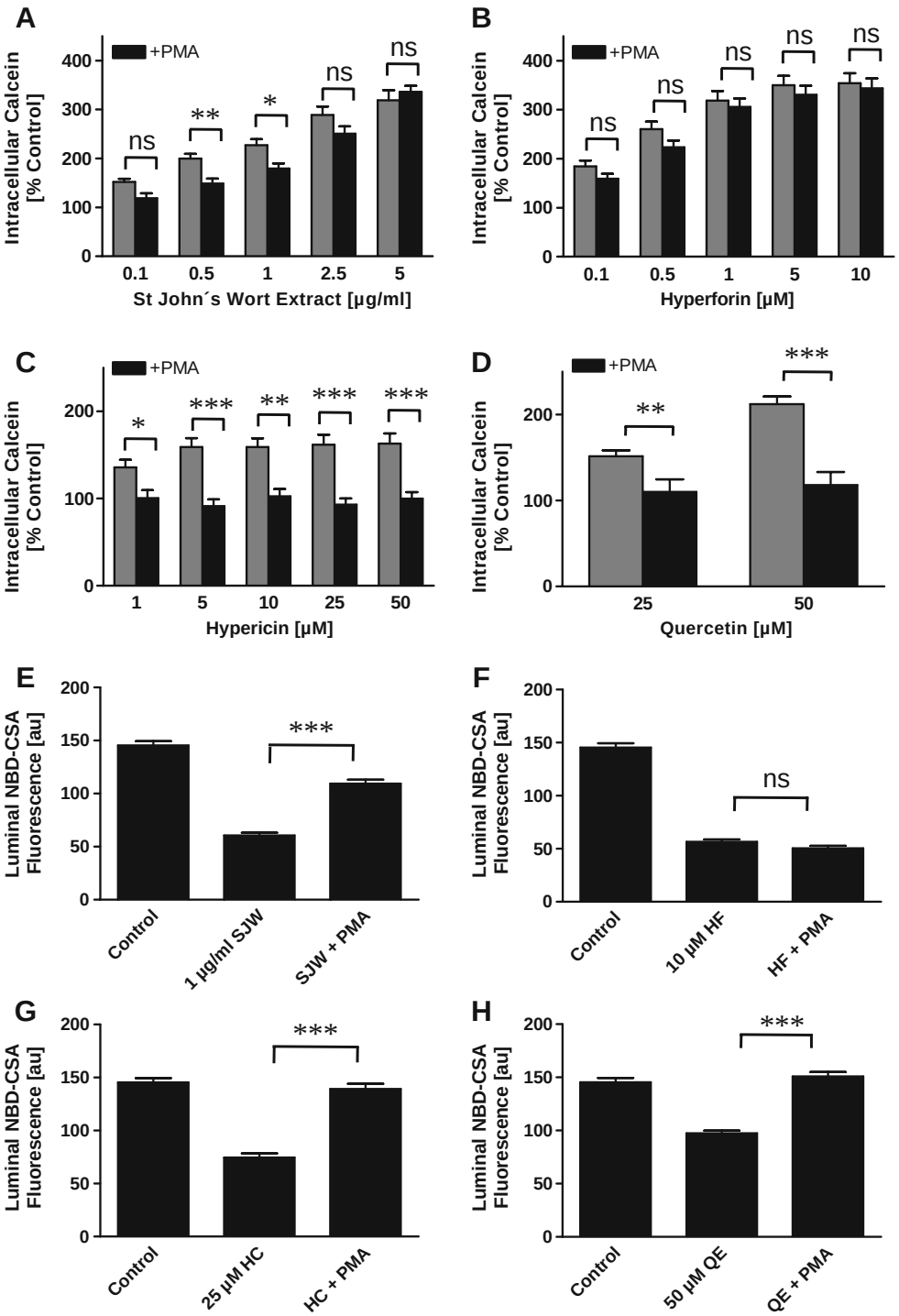

Fig. 6. PKC involvement in SJW-mediated modulation of P-glycoprotein activity. Effect of St. John's Wort extract (**A**), hyperforin (**B**), hypericin (**C**), and quercetin (**D**) on intracellular calcein accumulation in PBCEC with or without the PKC activator, PMA. Effect of St. John's Wort extract (**E**), hyperforin (**F**), hypericin (**C**), and quercetin (**D**) on luminal NBD-CSA fluorescence in pig brain capillaries with or without PMA. Data for calcein assays (**A**–**D**) are means ± SEM (n=6). Data for luminal NBD-CSA fluorescence (**E**–**H**) are means ± SEM for 5–8 capillaries from a single preparation (pooled tissue from 5 to 6 pig brains). Units are arbitrary fluorescence units (0–255). Statistics: *p<0.05; **p<0.01; ***p<0.001, significantly different from controls (Student's t-test).

further experiments, we preincubated isolated pig brain capillaries with SJW extract, hyperforin, hypericin, or quercetin with and without 10 nM PMA, followed by exposure to NBD-CSA to visualize P-glycoprotein-mediated transport. In these experiments, PMA partly counteracted the effect of SJW extract (Fig. 6E) but had no effect on the hyperforin-mediated decrease of luminal NBD-CSA fluorescence (Fig. 6F). However, the hypericin- and quercetin-induced decreases in luminal fluorescence were completely abolished by PMA (Fig. 6G and H), confirming our data obtained from PBCEC.

These findings suggest that hyperforin acted through a PKC-independent mechanism to reduce P-glycoprotein function and that the effect observed with SJW extract was, at least in part, mediated through PKC. Our data also suggest that hypericin and quercetin acted through a PKC-dependent mechanism to reduce P-glycoprotein transport activity.

Quercetin Enhances P-glycoprotein Membrane Trafficking by Signaling Through PKC

The phenomenon of P-glycoprotein activation through low concentrations (<1 μM) of quercetin that we observed was studied in more detail in PBCEC and isolated capillaries. Our experiments in PBCEC revealed that the decrease of intracellular calcein fluorescence induced by 1 μM quercetin could be blocked by adding 10 nM BIM (Fig. 7A), whereas addition of PMA had no additive, synergistic effect. In isolated pig brain capillaries, we observed the same results (Fig. 7B). BIM abolished the quercetin-mediated increase of luminal NBD-CSA fluorescence (increase of P-glycoprotein transport activity). As in cultured PBCEC, PMA did not reverse the quercetin effect on P-glycoprotein.

The stimulatory effect on P-glycoprotein function that we found with low quercetin concentrations could have been due to increased vesicular trafficking of transporter protein to the plasma membrane. To test this hypothesis, we used brefeldin A (BFA), an inhibitor of vesicular trafficking that by itself had no effect on P-glycoprotein transport function (Fig. 7C and D). Fig. 7C shows that exposing PBCEC to 1 μM quercetin or 10 nM PMA decreased intracellular calcein fluorescence (increased P-glycoprotein transport activity) and that this effect was completely abolished by blocking vesicular trafficking with 5 μg/ml BFA. These data were confirmed in experiments with isolated capillaries (Fig. 7D) where BFA blocked the quercetin- and PMA-mediated increase in luminal NBD-CSA fluorescence (increased P-glycoprotein transport activity). Together, these findings indicate that similar to PMA, quercetin at low concentrations stimulated P-glycoprotein transport

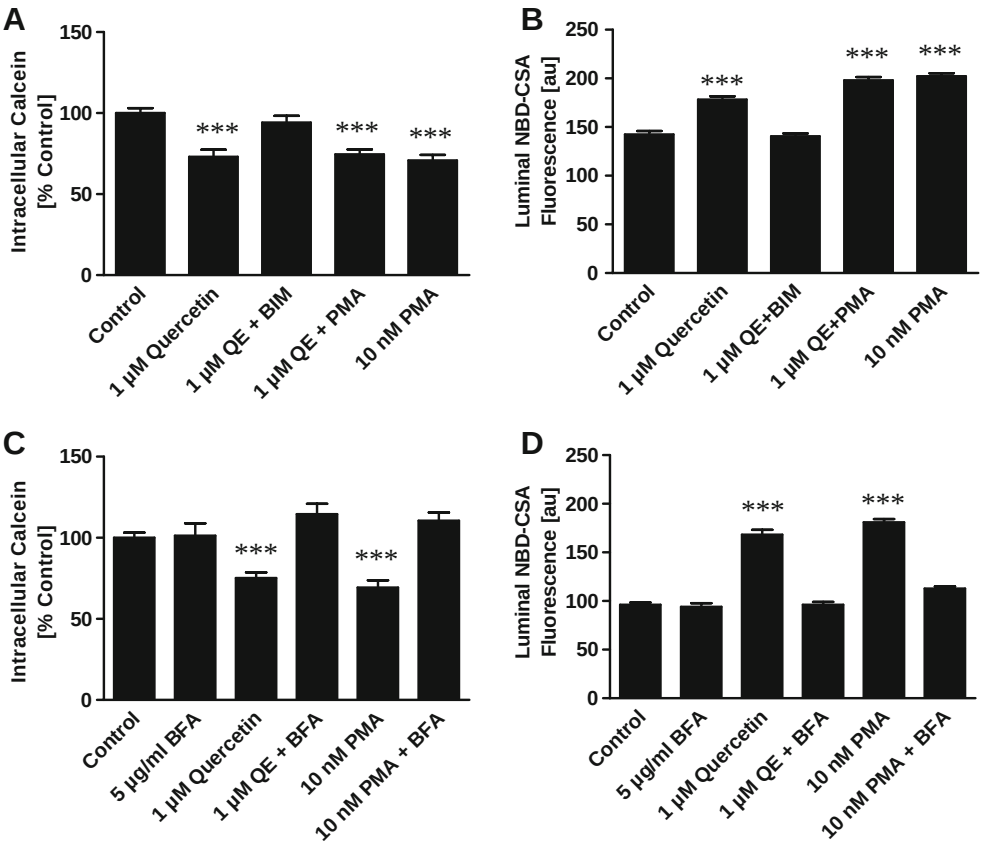

Fig. 7. Quercetin enhances P-glycoprotein membrane trafficking by signaling through PKC. BIM reversed the effect of quercetin and PMA on intracellular calcein fluorescence in PBCEC (**A**) and on luminal NBD-CSA fluorescence in pig brain capillaries (**B**). Brefeldin A, (BFA), an inhibitor of vesicular trafficking, also reverses the effect of quercetin and PMA on intracellular calcein fluorescence in PBCEC (**C**) and on luminal NBD-CSA fluorescence in pig brain capillaries (**D**). Data for calcein assays (**A** and **C**) are means ± SEM (n=6). Data for luminal NBD-CSA fluorescence (**B** and **D**) are means ± SEM for 5–8 capillaries from a single preparation (pooled tissue from 5 to 6 pig brains). Units are arbitrary fluorescence units (0–255). Statistics: ***$p<0.001$, significantly different from controls (Student's t-test).

function through increased vesicular trafficking of transporter protein to the membrane. Our data further suggest that this effect was mediated through PKC activation.

DISCUSSION

In the present study we investigated the short-term effect of SJW on P-glycoprotein transport activity. We show that SJW extract and the SJW constituents hyperforin, hypericin, and quercetin decreased P-glycoprotein activity in a dose- and time-dependent manner. Our data indicate that SJW extract and hyperforin directly inhibited P-glycoprotein-mediated transport, whereas hypericin and quercetin indirectly modulated the transporter through a mechanism involving protein kinase C (PKC). Quercetin displayed a biphasic effect; P-glycoprotein activity was increased at low quercetin concentrations but decreased at high concentrations. The quercetin-mediated increase in P-glycoprotein activity was likely due to trafficking and membrane insertion of sub-apical vesicles containing P-glycoprotein. Our findings are discussed in more detail in the following.

Short-term incubation with SJW extract, hyperforin, hypericin and quercetin revealed a dose- and time-dependent decrease in P-glycoprotein transport activity; these effects were less pronounced compared to PSC833, a P-glycoprotein-specific inhibitor. On the other hand, the SJW effects were significantly higher than those observed in recent studies using primary PBCEC cultures or LS-180 V and Caco-2 cell lines (13,14). This difference in effect might be due to the different species that were used (pig *vs*. human) or to differences in the composition of the SJW extracts, which depends on the extraction method that was used (30).

We also studied the mechanism through which SJW constituents modulated P-glycoprotein transport activity. Hypericin and quercetin have been described to affect protein kinase C (15,16), and it is known that PKC is involved in short-term regulation of P-glycoprotein. Based on this, we examined the possibility of SJW modulating P-glycoprotein through PKC. In our study, PKC activation with PMA decreased intracellular calcein fluorescence and increased luminal NBD-CSA fluorescence, indicating increased P-glycoprotein transport activity. Accordingly, PKC inhibition with BIM reduced P-glycoprotein-mediated transport and abolished the PMA-induced increase of P-glycoprotein activity. This finding is in agreement with previous studies, which show that treatment of several cell types with PKC activators enhanced P-glycoprotein activity and drug efflux, and, conversely, treatment with PKC inhibitors decreased drug transport activity (18,19,31). However, our results are in contrast to recent findings by Hartz *et al.*, who showed in rat brain capillaries that short-term exposure (1 h) to PMA resulted in a concentration-dependent decrease in steady-state luminal accumulation of NBD-CSA; in these experiments, reduced P-glycoprotein transport activity was reversed with BIM. This opposite effect of PMA and BIM on P-glycoprotein in rat compared to pig may be explained by the use of Ca^{2+}-containing buffer in the present study *versus* Ca^{2+}-free buffer by Hartz *et al.* (17). It is likely that in our experiments Ca^{2+}-dependent, classical PKC isoforms were activated, whereas Ca^{2+}-independent PKC isoforms may have been involved in the signaling observed by Hartz *et al.* Moreover, PKC regulation of P-glycoprotein is complex and has not been fully elucidated, thus, yet unknown intermediate steps between the kinase and the transporter could be involved, which might also explain the opposite effects observed by Hartz *et al.* and us. In addition, Hartz *et al.* and Bauer *et al.* recently showed that blood-brain-barrier P-glycoprotein underlies context-dependent regulation (32,33). In their studies, the authors demonstrated that in a P-glycoprotein regulatory cascade, the same signaling protein acted differently depending on the stimulus. Others have made similar observations and demonstrated in different tumor cell lines that the PMA effect on P-glycoprotein and drug accumulation varied (34). There are also at least eight PKC isoforms that could contribute to the species differences described above (35). For example, treatment of tumor cell lines with PMA affected PKCα but not PKCζ (18). A similar scenario might be the case here, but details remain to be elucidated.

We also investigated the link between PKC and SJW-induced P-glycoprotein modulation and found that the hyperforin-mediated decrease of P-glycoprotein transport activity was not abolished by activating PKC with PMA. This indicates that hyperforin inhibited transporter function through direct interaction. In contrast, the decrease in P-glycoprotein activity after exposure to hypericin and quercetin was completely blocked with PMA. This suggests that these two compounds modulated P-glycoprotein function through inhibiting PKC, which is in agreement with previous studies showing PKC inhibition with both compounds (15,36). However, some PKC inhibitors, such as staurosporin and calphostin-C, also bind to P-glycoprotein (37). Hence, the possibility exists that inhibition of P-glycoprotein function by hypericin and quercetin results either from directly interacting with the transporter or from inhibition of P-glycoprotein phosphorylation by direct or indirect effects on PKC. In accordance with our findings with hyperforin, hypericin and quercetin, PMA partially blocked the effect by SJW extract, which is a mixture of the compounds we studied. This could indicate a complex pharmacological effect with SJW extract.

For quercetin, a flavonol constituent of SJW, we made a particularly interesting observation. At concentrations up to 10 μM, quercetin reduced intracellular calcein levels in PBCEC and increased luminal NBD-CSA fluorescence in pig brain capillaries, indicating increased P-glycoprotein activity. Only at higher concentrations (25–50 μM), quercetin increased calcein fluorescence in PBCEC and decreased NBD-CSA accumulation in capillary lumens, indicating a decrease in P-glycoprotein transport function. Such a concentration-dependent biphasic effect of quercetin has been described previously by Mitsunaga *et al.* in mice *in vivo* (31). These authors showed that brain-to-plasma concentration ratios of the P-glycoprotein substrate vincristine were decreased in mice after administration of 0.1 mg/kg quercetin. Dosing mice with 1.0 mg/kg quercetin, however, resulted in increased vincristine brain-to-plasma concentration ratios (31). Thus, these *in vivo* findings also suggest P-glycoprotein activation at low quercetin doses and transporter inhibition at high quercetin doses. Since PBCEC and brain capillary exposure to quercetin was short in our experiments (maximum of 60 min), it is unlikely that the initial activation of P-

glycoprotein activity was due to transcriptional activation and increased protein levels. It is possible, however, that low concentrations of quercetin activated PKC, resulting in increased P-glycoprotein phosphorylation and enhanced transporter function, as has been described previously (31). Indeed, we found that PKC inhibition with BIM completely abolished the quercetin-induced increase in P-glycoprotein transport activity. In this regard, it has been reported that PMA, similar to diacylglycerol, has a dual effect on PKC in that it activates the kinase over short exposure times, whereas long-term PMA exposure inactivates PKC (35). Thus, it is possible that quercetin could also affect PKC in such a manner.

We also observed that quercetin-mediated activation of P-glycoprotein was sensitive to brefeldin A (BFA). This is an interesting finding because BFA is an inhibitor of vesicular trafficking (38,39), which suggests that quercetin affects P-glycoprotein movement to the membrane. This is in agreement with studies by Fu and Labroille who demonstrated in HeLa and K562 MDR cell lines that in addition to localization in the plasma membrane, P-glycoprotein is also present in sub-apical vesicles that can be rapidly inserted into the membrane. Likewise, Hartz and colleagues have recently proposed that at the blood-brain barrier, PKC may influence P-glycoprotein trafficking as a means of rapidly modulating transporter activity (17).

CONCLUSION

The data presented here provide important new insights into the mechanism of how SJW constituents interact with P-glycoprotein at the blood-brain barrier. Potentially, our findings have several consequences for CNS therapy. First, quercetin in SJW medications could activate PKC *in vivo* and increase P-glycoprotein-mediated efflux transport at the blood-brain barrier, which in turn could have negative effects on CNS therapy. In this regard, Schulz *et al.* (40) demonstrated that after a single oral dose of 600 mg SJW extract, quercetin plasma levels reached about 0.1 μM, a concentration below those used in the present study. Thus, it remains to be determined if quercetin affects blood-brain-barrier P-glycoprotein *in vivo*. Second, studies by other groups have demonstrated that therapeutic doses of SJW extract resulted in hyperforin plasma levels between 200 and 800 nM in humans (41), concentrations at which hyperforin has been shown to inhibit P-glycoprotein over the short-term present study, (11). In this regard, other pharmacokinetic studies in humans demonstrated that after oral administration of SJW extract, plasma levels ranged from 0.8–2.6 μM for hyperforin and from 0.03 to 0.2 μM for hypericin (41–43). Note that these clinically observed plasma concentrations after oral application of SJW extract are in the range of those used in the present study. Thus, these findings and our data suggest that hyperforin and hypericin in over-the-counter SJW preparations could potentially affect P-glycoprotein and cause adverse CNS side effects or drug-drug interactions at the level of the blood-brain barrier in patients using SJW. However, additional pharmacokinetic and/or pharmacodynamic factors may influence SJW effects in humans *in vivo,* and therefore, the clinical relevance of our findings is not clear and remains to be demonstrated.

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CONFLICTS OF INTEREST None.

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