Short communication

FLAVONOID TRANSPORT ACROSS RBE4 CELLS: A BLOOD-BRAIN BARRIER MODEL

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Abstract: There is a growing interest in dietary therapeutic strategies to combat oxidative stress-induced damage to the Central Nervous System (CNS), which is associated with a number of pathophysiological processes, including Alzheimer's and Parkinson's diseases and cerebrovascular diseases. Identifying the mechanisms associated with phenolic neuroprotection has been delayed by the lack of information concerning the ability of these compounds to enter the CNS. The aim of this study was to evaluate the transmembrane transport of flavonoids across RBE-4 cells (an immortalized cell line of rat cerebral capillary endothelial cells) and the effect of ethanol on this transport. The detection and quantification of all of the phenolic compounds in the studied samples (basolateral media) was performed using a HPLC-DAD (Diode Array Detector). All of the tested flavonoids (catechin, quercetin and cyanidin-3-glucoside) passed across the RBE-4 cells in a time-dependent manner. This transport was not influenced by the presence of 0.1% ethanol. In conclusion, the tested flavonoids were capable of crossing this blood-brain barrier model.

Key words: Anthocyanin, Blood-brain barrier, Flavonol, 3-flavanol, RBE4, Transport

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INTRODUCTION

The results of several epidemiological studies have provided support for the association between better health and the consumption of fruit, vegetables and certain beverages rich in polyphenols, such as tea and red wine [1, 2]. Several polyphenols have been demonstrated to have clear antioxidant properties *in vitro*, as they can act as chain breakers or radical scavengers depending on their chemical structure [3, 4]. Numerous studies have indicated that a high consumption of fruits and vegetables is associated with a reduced incidence of age-associated illnesses, including neuro-degenerative diseases like Alzheimer's disease, amyotrophic lateral sclerosis and Parkinson's disease [5, 6]. Despite this association, there is little information regarding the interaction between flavonoids and the blood-brain barrier (BBB).

Quercetin is the main flavonol in our diet, and it is present in many fruits, vegetables and beverages. It is abundant in onions (0.3 mg/g fresh weight) and tea (10-25 mg/l) [7, 8]. The main 3-flavanols found in our diet are catechin, epicatechin, gallocatechin and epigallocatechin, which are very abundant in tea: a green tea infusion contains about 1 g/l of 3-flavanols [9] and the content in black tea is about half this value [10]. Other sources are red wine (270 mg/l) [11] and chocolate [12]. In the human diet, anthocyanins are most abundant in red fruit such as cherries, plums, strawberries, raspberries, blackberries, grapes, red currants and black currants. Wine contains approximately 200-350 mg anthocyanins/l, and these anthocyanins are transformed into various complex structures as the wine ages [13, 14].

Due to their abundance and properties, the transport of these phenolic compounds across the BBB is of great interest. In this study, we investigated the transmembrane transport of flavonoids across RBE-4 cells (rat cerebral capillary endothelial cells) and the effect of ethanol on this transport.

MATERIALS AND METHODS

Reagents

(+)-catechin, quercetin, formic acid, Minimum Essential Medium, Ham's F10, neomycine, penicillin G, amphotericin B, streptomycin, HEPES and trypsin-EDTA were purchased from Sigma-Aldrich® (Madrid, Spain). Fetal bovine serum, basic fibroblast growth factor and HBSS were from Gibco (Barcelona, Spain), and cyanidin-3-glucoside was from Extrasynthase (Lyon, France).

Flavonoid analysis

Catechin and quercetin were analysed by HPLC in the Elite Lachrom system (L-2130) on a 150 x 4.6-mm i.d. reversed-phase C18 column (Merck, Darmstadt). The detection was carried out using a diode array detector (L-2455). The solvents were A: H_2O/HCOOH (9.9:0.1), and B: CH_3CN. For catechin analysis, the program started with 93% A and 7% B for 4 min, and had a gradient of 7-25% B over 46 min at a flow rate of 0.5 ml/min. For quercetin,

the program initiated with 95% A and 5% B and had a gradient of 5-25% B over 46 min at a flow rate of 0.5 ml/min. The column was washed with 100% B for 10 min and then stabilized at the initial conditions for another 10 min.

Cyanidin-3-glucoside was analysed by HPLC in the Elite Lachrom system (L-2130) on a 250 x 4.6-mm i.d. reversed-phase C18 column (Merck, Darmstadt). The detection was carried out at 520 nm using a diode array detector (L-2455). The solvents were A: H_2O/HCOOH (9:1), and B: H_2O/HCOOH/CH_3CN (6:1:3). The gradient consisted of 20-55% B for 50 min at a flow rate of 1.0 ml/min. The column was washed with 100% B for 10 min, and then stabilized at the initial conditions for another 10 min.

Cell and culture conditions

The RBE4 cell line was supplied by Dr. Francoise Roux (INSERM U. 26, Hôpital Fernand Widal, Paris, France). The RBE4 clone was maintained in a humidified atmosphere of 5% CO_2, 95% air at 37ºC. These cells (passages 64-70) were grown in Minimum Essential Medium/Ham's F10 (1:1) supplemented with 300 μg/ml neomycine, 10% fetal bovine serum (FBS), 1 ng/ml basic fibroblast growth factor, 100 U/ml penicillin G, 0.25 mg/ml amphotericin B, 100 mg/ml streptomycin, and 25 mM HEPES. The cell medium was changed every 48 h, and the cells reached confluence after 6 to 7 days of culture. For subculturing, the cells were dissociated with 0.25% trypsin-EDTA, diluted 1:5 and subcultured in Petri dishes with a 21-cm^2 growth area (Corning Costar, Badhoevedorp, The Netherlands). For the experiments, the RBE4 cells were seeded on transwell inserts (collagen-coated polytetrafluoroethylene membrane, 0.4 μm pore size, 12 mm diameter, Corning Costar). The inserts were placed in 12-well plates. All the experiments were performed 9 to 10 days after the initial seeding.

Transport studies

The transepithelial electrical resistance (TEER) of cells grown in transwells was measured using an epithelial voltohmmeter fitted with planar electrodes (EVOM; World Precision Instruments, Stevenage, UK). TEER was monitored before and after the experiments: only the results obtained with cell monolayers that maintained TEER > 100 Ω/cm^2 were considered. The medium was removed and the cells were washed with HBSS medium with 1.0 mM $MgCl_2$ and 0.25 mM $CaCl_2$ at pH 7.4. A flavonoid solution in HBSS with 0.1% FBS was added to the apical side of the cells and the same medium free of polyphenols was added to the basolateral compartment. Transepithelial transport was followed as a function of time, at 37ºC. Samples were taken from the basolateral side and replaced with fresh medium. The samples were frozen until used for the HPLC analysis. The transport efficiency is given as a percentage, calculated according to: (the compound concentrations on the basolateral side at a given time)/(compound concentrations on the apical side at the zero hour)*100, as in [15].

Statistical analysis

All the experiments were conducted at least in triplicate. The values are expressed as the arithmetic mean ± SEM. The statistical significance of each treatment was evaluated via one-way analysis of variance (ANOVA) followed by the Bonferroni test. The difference between the treatment in the absence or presence of ethanol was evaluated via two-way analysis of variance (ANOVA) followed by the Bonferroni test. Differences were considered significant when $p < 0.05$.

RESULTS AND DISCUSSION

Flavonoid transport through RBE-4

We studied three of the most common polyphenols in the human diet. These molecules belong to different classes of polyphenols, namely flavonols (quercetin), 3-flavanols (catechin) and anthocyanins (cyanidin-3-glucoside), but they share a structural feature: the presence of a catechol group in the B ring (Fig. 1).
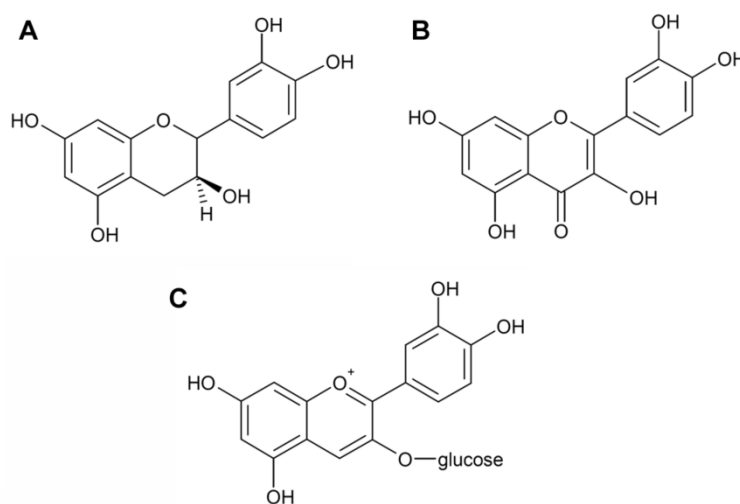

Fig. 1. The chemical structures of A – (+)-catechin, B – quercetin, and C – cyanidin-3-glucoside (flavylium form).

Although there are some reports demonstrating some of the biological effects of these polyphenols [16], there is a lack of information on the transport of these compounds, and their ability to cross the various barriers existing in the organism. The intestinal barrier is the first obstacle faced by the compounds that enter the organism orally. There are reports showing that flavonoids are able to cross the intestinal barrier and can be found in the plasma [17-20]. It is reasonable to assume that these compounds are able to reach the blood-brain barrier, and, if able to cross it, gain access to the central nervous system.

A previous study performed with some flavonoids and their metabolites in a similar model indicated that certain flavonoids did cross the BBB [21]. In this study, the tested flavonoids were able to cross RBE4 cells, a model for the BBB [22]. Catechin was found in the basolateral side in concentrations that reached 18% of the apical concentration (Fig. 2).
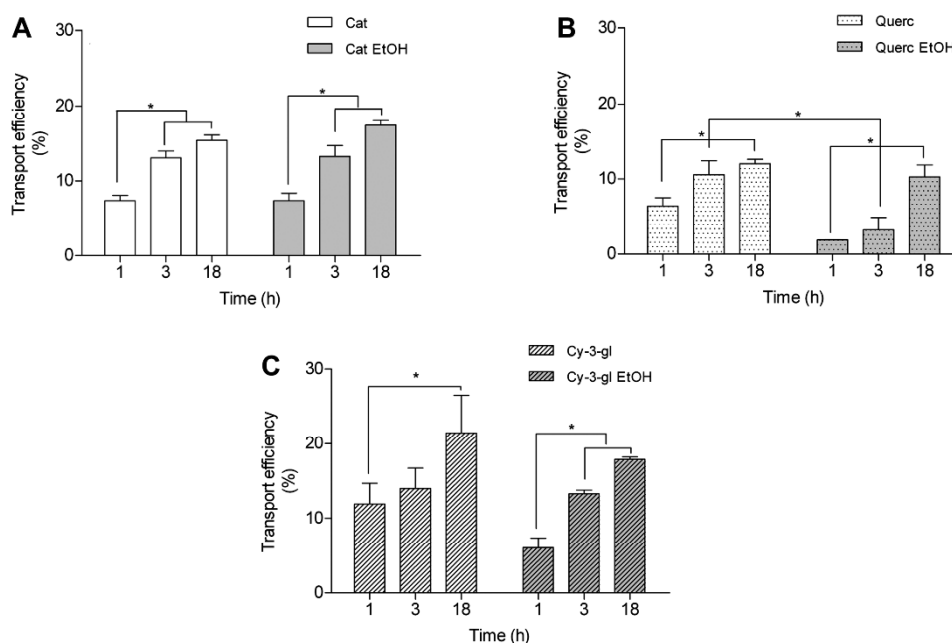

Fig. 2. The transport efficiency of flavonoids across RBE4 cells (Apical → Basolateral) in the absence and presence of 0.1% ethanol. The results are presented as the transport efficiency (%; mean ± SEM). The transport efficiency was calculated based on (compound concentrations on the basolateral side at a given time)/(compound concentrations on the apical side at the zero hour)x100. A – (+)-catechin (30 μM); B – quercetin (30 μM); C – cyanidin-3-glucoside (100 μM). *significantly different with $p < 0.05$.

Quercetin was also transported across the RBE4, but to a lesser extent. Nevertheless, it reached 14% after only 3 h of incubation. Cyanidin-3-glucoside demonstrated the greatest ability to cross the barrier. This flavonoid has a particular feature: the presence of a glucose moiety in the C ring. While for catechin and quercetin, the hypothesis of transport by diffusion can be raised due to their hydrophobicity, for cyanidin-3-glucoside, this hypothesis is not as viable due to the presence of the glucose moiety. It is highly improbable that the glucose molecule would be able to cross the cell membrane without a transporter system. In the case of cyanidin-3-glucoside, GLUT1 is a possible transporter, concurrent with the recent finding that intestinal GLUT2 may be involved in the transport of these compounds [23]. The role of efflux transporters, which control

xenobiotic flux across the BBB, has to be taken into consideration. The efflux transporters such as P-glycoprotein (Pgp), multidrug resistance-associated proteins (MRPs) and the breast cancer resistance protein (BCRP) play a role in limiting drug entry into the CNS. However, there is no direct information on its role in limiting flavonoid entry [21, 24].

The influence of ethanol on flavonoid transport
Ethanol is also a component of the diet, especially among wine consumers. The three studied flavonoids are found to some extent in wine. Furthermore, there are some reports that show the influence of ethanol on the bioavailability of flavonoids [24], allegedly because it may facilitate flavonoids crossing the cell membrane. Therefore, the effect of ethanol in the transport of flavonoids across the BBB was also studied (Fig. 2). There was no significant effect of ethanol on the flavonoid transport. The transport decreased in the case of quercetin and had a tendency to decrease in the case of cyanidin-3-glucoside.

CONCLUSION

Catechin, quercetin and cyanidin-3-glucoside were able to cross RBE4 cells. The transport was time-dependent. Ethanol did not have any significant influence on this transport. Thus, these results suggest transcellular transport of these flavonoids across the BBB *in vivo*, possibly explaining some of the biological effects already demonstrated [25, 26].

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