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Cellular Uptake and Efflux of *trans*-Piceid and Its Aglycone *trans*-Resveratrol on the Apical Membrane of Human Intestinal Caco-2 Cells

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Two stilbenes (*trans*-piceid and its aglycone *trans*-resveratrol) were investigated in the uptake across the apical membrane of the human intestinal cell line Caco-2 in order to determine their mechanisms of transport. The uptake was quantified using a reverse phase high-performance liquid chromatography method with fluorescence detection. The rate of cellular accumulation in the cells was found to be higher for *trans*-resveratrol than for *trans*-piceid. In addition, *trans*-resveratrol uses passive transport to cross the apical membrane of the cells, whereas the transport of *trans*-piceid is likely active. With regard to the mechanisms of transport, the involvement of the active transporter SGLT1 in the absorption of *trans*-piceid was deduced using various inhibitors directly or indirectly exploiting the activity of this transporter (glucose, phlorizin, and ouabain). Moreover, we investigated the involvement of the multidrug-related protein 2 (MRP2), an efflux pump present on the apical membrane, in stilbene efflux by Caco-2 cells. The effect of MK-571 (an MRP inhibitor) seems to implicate MRP2 as responsible for apical efflux of *trans*-piceid and *trans*-resveratrol.

KEYWORDS: Caco-2 cells; *trans*-piceid; *trans*-resveratrol; apical transporters; SGLT1; MRP2

INTRODUCTION

Several epidemiological studies have shown that a moderate consumption of red wine is related to a reduced incidence of heart disease and cancer (*1*). This so-called "French paradox" has been related to the polyphenolic constituents present in high levels in red wine (*2*). Hydroxystilbenes have been found in a number of plant species, but grapes and wine are probably the most important foods containing these substances (*3*). In red wine, stilbene levels usually reach concentrations up to 20 mg/L and the most significant compound in this group in terms of possible positive health benefits is the *trans*-resveratrol (*trans*-3,5,4′-trihydroxystilbene) (*4*). Indeed, resveratrol has been reported to have potential cancer chemopreventive activities (*5*, *6*) and may exert a protective effect against atherogenesis through its antioxidant properties (*7*).

However, wine has a low level of aglycone (*trans*-resveratrol) as compared to its glucoside *trans*-piceid. Indeed, the amount of *trans*-piceid in red wine may be more than 10 times greater than that of its aglycone *trans*-resveratrol (*8*, *9*). *trans*-Resveratrol seems to have a greater biological effect than the glycoside *trans*-piceid (*7*, *10*), but hydrolysis of these glycosylated derivatives by a β-glucosidase can occur in human small intestine and liver, as reported by Day et al. (*11*) for flavonoid and isoflavonoid glucosides, which would enhance the quantity of *trans*-resveratrol available from the diet.

Although there is considerable evidence that stilbenes from red wine provide health benefits, there are few data about their absorption and bioavailability in humans. Recently, Soleas et al. (*12*) reported that oral administration of *trans*-resveratrol leads to a high absorption, resulting in a significant plasma bioavailability in rat. However, whether *trans*-resveratrol is metabolized to other compounds is not fully understood. Recently, we studied its distribution in organs and tissues after administration of this ^{14}C-labeled compound in the mouse. The results showed that intact resveratrol and its metabolites (glucuronides and/or sulfated derivatives) are detected in liver 3 h after oral administration (*13*). These results corroborate our previous studies showing that *trans*-resveratrol can be glucuronidated in human and rat liver microsomes (*14*). Moreover, Kuhnle et al. (*15*) showed the same metabolism during uptake of *trans*-resveratrol across the small intestine of rat.

The intestinal epithelium is the first biological barrier to be crossed by polyphenols, so better knowledge of the specific intestinal cell mechanisms involved is of interest when assessing the benefit associated with wine consumption. A recent study has shown that *trans*-resveratrol is efficiently absorbed across the intestinal Caco-2 cells and that this absorption increases with

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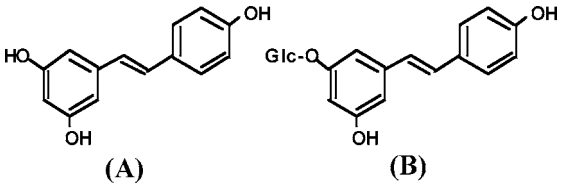

Figure 1. Chemical structures of (**A**) *trans*-resveratrol and (**B**) *trans*-piceid.

higher concentrations (*16*). However, the mechanisms underlying the intestinal absorption of stilbenes are still unknown.

The aim of the present study was to investigate the mechanisms of intestinal uptake of two stilbenes (*trans*-piceid and its aglycone *trans*-resveratrol) using human intestinal Caco-2 cell monolayers. This cell line spontaneously differentiates into polarized cell monolayers with many enterocyte-like properties of transporting epithelia (*17*). This model has already been used to study the transepithelial transport of polyphenols (*18*). We examined the mechanism of apical transport of these two polyphenols and hypothesized the possible involvement of the transporter SGLT1 (sodium-dependent glucose transporter) and the MDR (multidrug resistance protein) efflux transporters. To this end, we investigated the effects of several inhibitors on the characteristics of intestinal transport.

MATERIALS AND METHODS

Chemicals. *trans*-Resveratrol (**Figure 1A**), phlorizin, ouabain, 2-deoxyglucose, sodium azide, D-glucose, 2,4-dinitrophenol (DNP), verapamil, and indomethacin were purchased from Sigma-Aldrich (St. Quentin Fallavier, France). MK-571 was purchased from Tebu-bio (Le Perray-en-Yvelines, France). *trans*-Piceid (**Figure 1B**) was extracted, purified from *Vitis vinifera* cell suspension cultures, and identified using spectroscopic methods as previously described (*19*). All of the solvents were purchased in high-performance liquid chromatography (HPLC) grade quality (Scharlaud, Barcelona, Spain).

Cell Culture. Human colon adenocarcinoma Caco-2 cells, obtained from the American Type Culture Collection (Molsheim, France), were cultured in high Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 1% L-glutamine, 100 units/mL penicillin, and 100 μg/mL of streptomycin (Sigma-Aldrich). All cells were grown to confluence at 37 °C in a humidified atmosphere of 5% CO_2−95% air and subcultured using 0.02% ethylenediaminetetraacetic acid and 0.05% trypsin.

Uptake Studies. For cellular uptake studies, Caco-2 cells (at passages 30−50) were seeded on 12 well plates at a density of 9×10^4 cells/cm^2. The culture medium was replaced every 2 days and 1 day before uptake studies. Experiments were conducted 12−14 days postconfluence. Before experiments, the culture medium was removed and the cells were quickly rinsed with transport buffer consisting of (mM) NaCl (140), KCl (5.33), $CaCl_2$ (1.26), $MgSO_4$ (0.84), KH_2PO_4 (0.44), and mannitol (5.48) buffered at pH 7.4. Monolayers were immediately incubated twice for 30 min with transport buffer prewarmed at 37 °C. In some experiments, transport inhibitors were included in the final 30 min preincubation. Then, the preincubation buffer was removed and the cells were incubated with 800 μL of the different stilbenes (150−300 μM) dissolved in transport buffer/DMSO (dimethyl sulfoxide) (99.5/0.5, v/v). At the end of the incubation period, the medium was immediately removed, the monolayers were rapidly washed twice with ice-cold transport buffer, and the cells were lysed with methanol. The cellular extracts were concentrated to dryness (40 °C), and the samples were dissolved in MeOH/H_2O (50/50, v/v) for HPLC analysis.

Efflux Experiments. For efflux measurement, Caco-2 cells were grown in 12 well plates and used on days 12−14 postconfluence. The monolayers were washed rapidly, preincubated twice with transport buffer (prewarmed at 37 °C) before the loading of 300 μM stilbenes in transport buffer, and incubated for 10 min at 37 °C. Thereafter, the efflux was monitored at 37 °C for different times (0, 15, 30, 45, 60, and 90 s) in 800 μL of transport buffer alone (*20*). At each time point, the medium was rapidly removed and the cell monolayers were washed twice with ice-cold buffer to stop all cellular activities. Then, the cells were scraped twice with methanol, and the amount of stilbenes recovered in the cells was measured in these methanolic extracts as described in Uptake Studies.

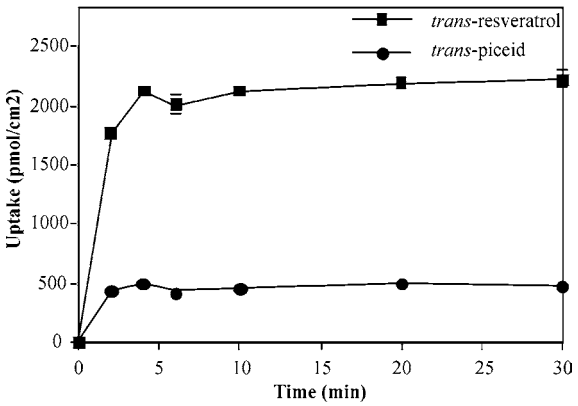

Figure 2. Time course of apical transport in Caco-2 cells. Uptake was measured for *trans*-resveratrol (■) and *trans*-piceid (●) at different time intervals. Values are expressed as means ± standard deviations ($n \geq 3$) in pmol of stilbenes accumulated in Caco-2 cell monolayers per cm^2 of epithelium.

HPLC Analysis. Before HPLC analysis, samples were filtered through Millipore filters (0.45 μm). HPLC was performed on a system equipped with a 250 mm × 4 mm Prontosil C_{18} (5 μm) reverse phase column (Bischoff Chromatography, Leonberg, Germany) protected by a guard column of the same material. Separation was performed at a flow rate of 1 mL/min with a mobile phase composed of (A) H_2O:TFA (99.9:0.1, v/v) and (B) ACN:TFA (99.9:0.1, v/v). The run was set as follows: 0−10 min, from 15 to 20% B; 10−16 min, 20% B; 16−18 min, from 20 to 22% B; 18−22 min, 22% B; 22−30 min, from 22 to 30% B; 30−32 min, from 30 to 100% B; 32−37 min, 100% B. Chromatographic peaks were monitored using a fluorescence detector (FD 500 Groton, Concord, United States) set at optimal wavelengths for detection of both *trans*-resveratrol and *trans*-piceid (λ_{exc}, 300 nm; λ_{em}, 390 nm) (*9*). Quantification of compounds was estimated from calibration curves that were prepared with authentic standards molecules.

Statistical Analysis. Data were expressed as the means ± standard deviations of 3−6 determinations. Statistical analysis was performed using Student's *t*-test, and $P < 0.05$ was considered to be significant.

RESULTS

Time Course of Stilbene Uptake by Caco-2 Cells. The uptake of *trans*-resveratrol and *trans*-piceid (150 μM) was measured for incubation times ranging from 2 to 30 min in Caco-2 cell monolayers 12−14 days postconfluence when the cells are fully differentiated (**Figure 2**). The results are expressed in pmol/cm^2, since the number of cells/cm^2 is constant in each experiment (data not shown). Whatever the time course, the uptake of *trans*-resveratrol was faster and greater than its glucoside. At 4 min, the accumulation rate of *trans*-resveratrol in Caco-2 cells was more than 4 times greater than that of *trans*-piceid (530 vs 120 pmol/min/cm^2, respectively). Moreover, a plateau was reached for *trans*-resveratrol and *trans*-piceid after about 4−6 min of incubation. Several hypotheses may account for this observation. First, the mechanisms of transport might have reached a saturation level, leading to a constant amount of stilbenes in the cells. However, incubation with higher concentrations of stilbenes (300 μM) increased their amount in the cells, so there was no saturation of the transport systems (data not shown). The second hypothesis is a metabolic reaction (glucuronidation, sulfation) concomitant with the uptake in the same proportions. HPLC analysis with metabolites standards

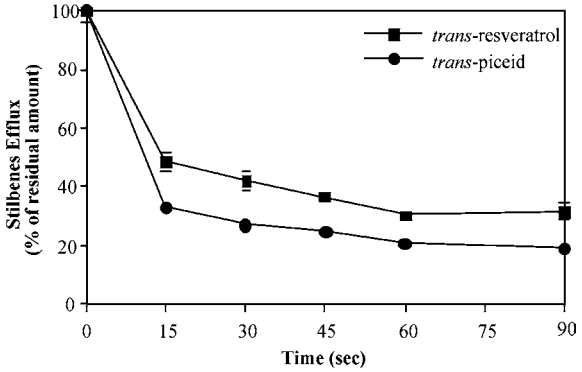

Figure 3. Time course of stilbene efflux from Caco-2 cells. Cells were preincubated for 10 min with *trans*-resveratrol (■) and *trans*-piceid (●), washed once with ice-cold transport buffer, and then incubated in transport buffer at 37 °C for different time intervals. Residual stilbenes present in the cells were expressed as means ± standard deviations ($n \geq 4$), and experimental values were normalized against control (time, 0 s) and expressed as percent.

(*trans*-resveratrol 3-*O*-glucuronide, *trans*-resveratrol 4′-*O*-glucuronide) (*14*) showed that except for the two initial stilbenes, no additional compounds were detected (data not shown). This suggests that these stilbenes may be weakly metabolized during the 30 min of the experiment. Thus, we hypothesized the occurrence of an apparent equilibrium between outward and inward transport.

Efflux Experiments. The hypothesis of a possible stilbene efflux from Caco-2 cells was analyzed with cells preloaded for 10 min with *trans*-resveratrol or *trans*-piceid (300 μM) and incubated in transport buffer alone for different times (0, 15, 30, 45, 60, and 90 s). The residual amount of stilbenes within the cells was examined by HPLC. The results reported in **Figure 3** show that only 48% of the incorporated *trans*-resveratrol and 32% of the incorporated *trans*-piceid were still present within the cells after 15 s of incubation, indicating a rapid efflux of the two compounds. After 90 s, there was only 31% of *trans*-resveratrol and 19% of *trans*-piceid in the cells, suggesting that the two molecules were extensively excreted by Caco-2 cells.

Drugs that cross the apical membrane may be substrates for apical efflux transporters, which extrude compounds back into the intestinal lumen (*21*). These apical efflux transporters are principally ABC proteins such as P-glycoprotein (P-gp) and multidrug resistance-associated protein 2 (MRP2), two membrane proteins involved in the active efflux of drugs and xenobiotics by limiting their bioavailability (*18*, *22*). The accumulation of *trans*-resveratrol or *trans*-piceid in the cells was measured in the presence of verapamil (P-gp inhibitor), MK-571, and indomethacin (MRP inhibitors). As shown in **Figure 4**, the presence of MK-571 (50 μM) and indomethacin (200 μM) increased the rate of cellular accumulation by 45 and 16% for *trans*-resveratrol and by 32 and 26% for *trans*-piceid, respectively. Verapamil (50 μM) did not significantly alter the accumulation of the two compounds. Moreover, the time course of stilbene uptake in the presence of MK-571 showed an increase in the accumulation of the two compounds, which became obvious with the increase of incubation time (**Figure 5**). These results suggest that MRP2 is involved in the efflux of *trans*-resveratrol and *trans*-piceid across the apical side of Caco-2 cells.

Study of Uptake Mechanisms. Subsequent experiments were done at time 4 min in the presence of MK-571 (50 μM) to limit the MRP2-mediated efflux. The effect of ATP depletion on the uptake of *trans*-resveratrol or *trans*-piceid was examined. The cells were preincubated for 30 min with either 5 mM DNP (2,4-dinitrophenol) or 15 mM sodium azide and 50 mM of 2-deoxyglucose. Then, the uptake of *trans*-resveratrol and *trans*-piceid was measured. The two metabolic inhibitors did not significantly affect the uptake of *trans*-resveratrol, whereas the uptake of *trans*-piceid was significantly reduced by about 15% with DNP and 30% with sodium-azide/2-deoxyglucose (**Figure 6**). These results suggest that *trans*-piceid is actively transported into Caco-2 cells via a carrier protein system.

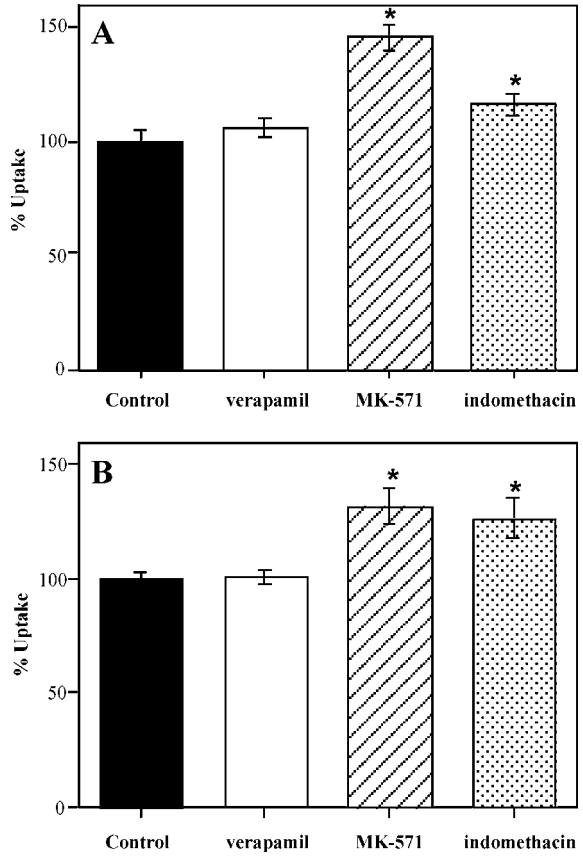

Figure 4. Inhibition of stilbene uptake in Caco-2 cells. Monolayers were incubated with *trans*-resveratrol ± inhibitors (**A**) or *trans*-piceid ± inhibitors (**B**) for 4 min. Data represent the means ± standard deviations of six determinations. Values were normalized against control (without inhibitor) and expressed as percent. * indicates values significantly different than control experiments ($P < 0.05$).

The sodium-dependent glucose cotransporter SGLT1 is the most abundant glucose transporter in the small intestine. Caco-2 cells have been reported to express SGLT1, so we tested whether SGLT1 was involved in the transport of *trans*-piceid across the apical membrane of Caco-2 cells. The cellular accumulation of this glucoside was measured in the cells in the presence of glucose (a substrate of SGLT1), phlorizin (a competitive inhibitor of this protein), or ouabaïn (which affects the Na^+ gradient-dependent process). As shown in **Figure 7**, the uptake of *trans*-piceid in the presence of 30 mM glucose, 0.5 mM phlorizin, or 5 mM ouabaïn was significantly lower than the control (32, 31, and 27% of inhibition, respectively). The same experiment was conducted for *trans*-resveratrol, but the inhibitors did not significantly influence the rate of transport (data not shown). These results suggest the involvement of SGLT1 in the uptake of *trans*-piceid across the apical side of Caco-2 cells.

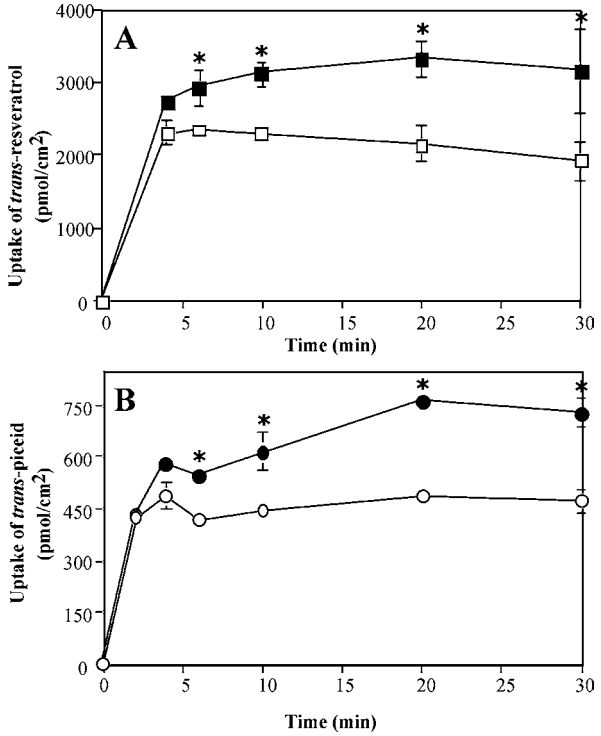

Figure 5. Time course of apical transport in Caco-2 cells. Uptake was measured for *trans*-resveratrol (**A**) and *trans*-piceid (**B**) at different time intervals. The monolayers were incubated for the indicated periods with (closed symbol) or without (open symbol) MK-571. Values are expressed as means ± standard deviations ($n \geq 3$) in pmol of stilbenes accumulated in Caco-2 cell monolayers per cm^2 of epithelium. * indicates values significantly different than control experiments ($P < 0.05$).

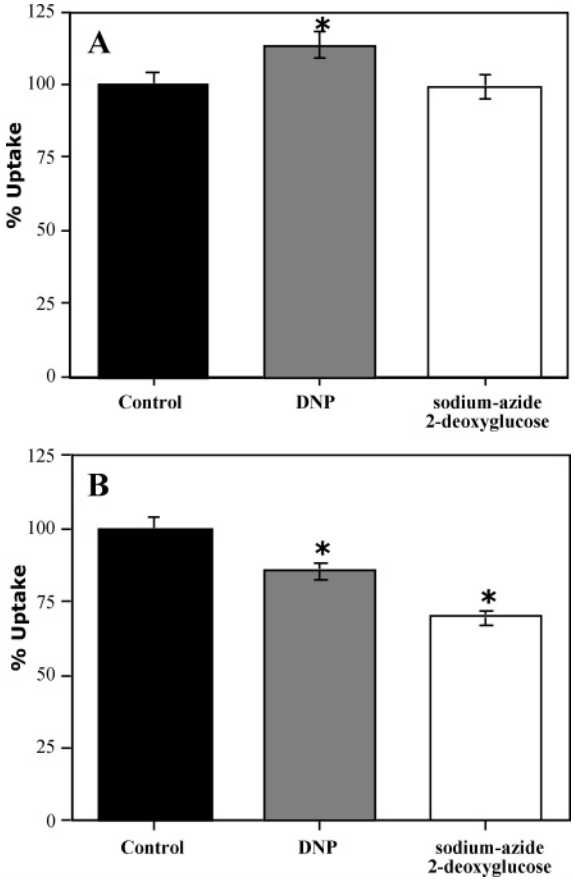

Figure 6. Effect of metabolic inhibitors on the uptake of stilbenes in Caco-2 cells. Monolayers were incubated with *trans*-resveratrol ± inhibitors (**A**) or *trans*-piceid ± inhibitors (**B**) for 4 min. Data represent the means ± standard deviations of six determinations. Values were normalized against control and expressed as percent. * indicates values significantly different than control experiments ($P < 0.05$).

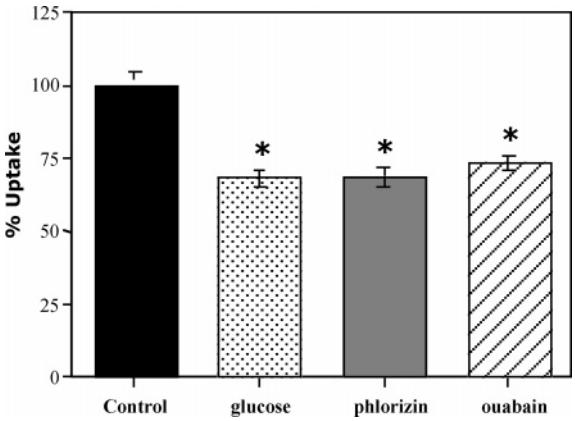

Figure 7. Inhibition of *trans*-piceid uptake in Caco-2 cells. Monolayers were incubated with *trans*-piceid ± inhibitors for 4 min. Data represent the means ± standard deviations of six determinations. Values were normalized against control values and expressed as percent. * indicates values significantly different than control experiments ($P < 0.05$).

DISCUSSION

It has previously been reported that phenolic aglycones (quercetin, genistein, daidzein) are taken up more easily and to a greater extent from the apical side of the Caco-2 cell monolayers than their glucosides (quercetin 4′-β-glucoside, genistin, daidzin) (*23*−*25*). In this work, we show for the first time that *trans*-resveratrol is absorbed across the apical membrane of Caco-2 cells more rapidly and in a higher amount than its glucoside *trans*-piceid. Murota et al. (*25*) suggest that affinity to the liposomal membrane plays an important role in the efficiency of cellular uptake by passive diffusion of lipophilic polyphenols. Consistent with these observations, we suggest that *trans*-resveratrol is absorbed across the apical membrane via a passive diffusion. Contrarily, *trans*-piceid is not absorbed via this transport system through the Caco-2 apical membrane, probably because of its lack of affinity toward the lipid bilayer of the cell surface. Indeed, Izumi et al. (*26*) have shown that isoflavone glucosides are very poorly absorbed as compared with aglycones in humans, probably due to their higher hydrophilicity and greater molecular weight. Moreover, our results clearly indicate that uptake of *trans*-piceid into Caco-2 cells occurs via the active transporter sodium-dependent SGLT1. This result is consistent with the hypothesis that the sugar transporter SGLT1, which is thought to be involved in the transport of flavonoïd glucosides (*27*), may also be capable of transporting this stilbene glucoside.

Stilbenes are present mainly as glycosides in red wine, but *trans*-resveratrol may be released from *trans*-piceid in the enterocytes by the hydrolytic action of β-glucosidases. There are two possible pathways by which glucosides might be hydrolyzed in the intestine (*28*). The first is cleavage by a cytosolic β-glucosidase (CBG), after passing the brush border membrane via SGLT1. Because we show that *trans*-piceid crosses the apical membrane of Caco-2 cells via this transporter, the latter mechanism may occur for *trans*-piceid. The second is deglycosylation on the luminal surface of the intestinal epithelium by the membrane-bound enzyme lactase phlorizin hydrolase (LPH), followed by passive diffusion of the released

aglycone. Hydrolysis by the small intestinal brush border glycoprotein LPH is the first step in the uptake of flavonoid glucosides by rat small intestine (*29*, *30*). Caco-2 cells have been reported to express the membrane-bound LPH on the apical side as well as the CBG. Although the expression of these two proteins in Caco-2 is weak (*28*, *31*), deglycosylation activity does occur in these cells (*32*). Indeed, our preliminary experiments indicate that *trans*-piceid is deglycosylated into *trans*-resveratrol by Caco-2 cells after 30 min of incubation (data not shown), but the mechanisms involved in this deglycosylation will require further investigations.

There is considerable evidence that *trans*-resveratrol absorbed in Caco-2 cells is subject to conjugation reactions yielding glucuronidated and sulfated metabolites, which can subsequently be exported to the luminal side of the cells (*16*, *33*). Moreover, an efflux of *trans*-resveratrol to the apical side of Caco-2 cells is thought to occur. MRP2 is highly expressed on the apical side of Caco-2, while the expression of MRP1 is minimal or undetectable as compared to MRP2 in these cells (*18*, *22*, *34*). Our results show the effective efflux of *trans*-resveratrol and *trans*-piceid, which was inhibited by MK-571, a selective inhibitor of the MRP isoforms. These observations suggest the involvement of MRP2 in the effective efflux of *trans*-resveratrol and *trans*-piceid mediated by this active carrier protein MRP2 across the apical side of the cells, although the involvement of MRP3 present at the basolateral membrane cannot be excluded.

In conclusion, our results suggest that the uptake of *trans*-resveratrol and *trans*-piceid is mediated by two different transport systems (a possible passive diffusion and active transport via SGLT1, respectively) and that MRP2 seems to be involved in their efflux. Work is in progress to determine the mechanism of transepithelial transport across the basolateral membrane using bicameral inserts, to assess the effective bioavailability of these two stilbenes.

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