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Resveratrol transport and metabolism by human intestinal Caco-2 cells

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Abstract

Resveratrol is a dietary constituent suggested to have protective effects against cancer as well as cardiovascular disease. The purpose of the study was to learn whether this agent could be absorbed in man and enter the systemic circulation. This was examined by measuring transport and metabolism of resveratrol (5–40 μM) by the human intestinal epithelial cell line Caco-2 cultured in Transwells. Transport across the Caco-2 monolayer occurred in a direction-independent manner with P_{app} values of $\approx 7 \times 10^{-6}$ cm s^{-1}, much higher than for the paracellular transport marker mannitol ($\approx 0.4 \times 10^{-6}$ cm s^{-1}), suggesting efficient absorption in-vivo. At the highest resveratrol concentration, the absorption increased, possibly due to saturation of metabolism. In sharp contrast to previous findings in the rat, the metabolism of resveratrol in Caco-2 cells involved mainly sulfation and, to a minor extent, glucuronidation. At low resveratrol concentrations, most of the sulfate conjugate was exported to the apical side, presumably by MRP2, which is well expressed in these cells. At high concentrations, there was a shift towards the basolateral side, possibly involving MRP3, which was recently shown also to be expressed in Caco-2 cells. These results indicate that absorption of resveratrol in-vivo may be high but with limited bioavailability due to efficient sulfate conjugation. Extensive accumulation of resveratrol in the Caco-2 cells, demonstrated in additional experiments, suggests enterocytes as a major target site for this cancer preventive agent.

Introduction

Resveratrol is a naturally-occurring polyphenolic compound with a distinct chemical structure (Figure 1), which has received much attention for its potential preventive actions in human disease (Jang et al 1997; Frémont 2000; Gusman et al 2001; Wu et al 2001). Red grapes and wines contain a considerable amount of resveratrol, 5–40 μM (Sato et al 1997; Frémont 2000). Thus, resveratrol, together with the bioflavonoid quercetin, has been suggested to be responsible for the lower prevalence of coronary heart disease observed among red wine drinkers. This has resulted in the widespread use of resveratrol in dietary supplements with doses in the 10–20 mg range. Potential mechanisms of action include inhibition of LDL oxidation, platelet aggregation and eicosanoid synthesis (Frémont 2000; Gusman et al 2001; Wu et al 2001). Moreover, resveratrol has been shown to have potential cancer preventive activity (Jang et al 1997; Zhao et al 1999; Gusman et al 2001). Mechanisms include actions on all three major stages of carcinogenesis (i.e. initiation, promotion and progression), with a variety of effects on signal transduction pathways (Ahmad et al 2001; Dörrie et al 2001; Lee & Safe 2001; She et al 2001; Yu et al 2001).

However, whether this polyphenol can gain access to proposed cellular sites of action via its dietary intake is not clearly understood. In the rat, it has been indicated that resveratrol may be reasonably well absorbed, although the bioavailability may be low (Andlauer et al 2000; Soleas et al 2001; Juan et al 2002). In man there is no information. Glucuronidation is of major importance in the metabolism of resveratrol in the rat, with a small contribution by sulfation (Andlauer et al 2000; Kuhnle et al 2000; Soleas et al 2001; Juan et al 2002). No bioavailability data for resveratrol exist in man, but both glucuronidation and sulfation of this polyphenol can occur (De Santi et al 2000a, b; Aumont et al 2001).

The objective of this pre-clinical study was to examine the transport and metabolism of resveratrol in the human intestinal cell line Caco-2, cultured as monolayers on Transwells. Previous studies have suggested this model to be a useful predictor of the

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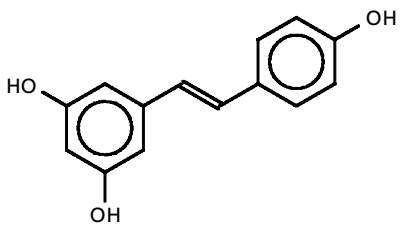

Figure 1 Chemical structure of resveratrol.

oral bioavailability of another polyphenol, the flavonoid chrysin, in-vivo (Walle et al 1999, 2001). There was clear transcellular absorption of resveratrol, but the bioavailability may be limited due to extensive sulfate conjugation.

Materials and Methods

Materials

Resveratrol (trans-3,4′,5-trihydroxystilbene) and aryl sulfatase from *Aerobacter aerogenes* were purchased from Sigma-Aldrich (St Louis, MO). Fetal calf serum was obtained from Summit Biotechnology (Ft Collins, CO) and other cell culture supplies from Mediatech Inc. (Fisher Scientific). [^{14}C]Mannitol (57 mCi mmol^{-1}) and sodium [^{35}S]sulfate (10–100 mCi mmol^{-1}) were purchased from Amersham Life Science Inc. (Arlington Heights, IL).

Cell culture

Caco-2 human colonic adenocarcinoma cells (ATCC) were cultured in Earle's Minimum Essential Medium with 1% nonessential amino acids, 10% fetal calf serum, penicillin and streptomycin. For transport experiments the cells (passage 60–80) were seeded 100 000 cells/insert on permeable polycarbonate inserts (0.4-μm pore size, Transwells, Costar) in 12-well clusters. Cell inserts were used for transport experiments at 18–28 days after seeding, when the transepithelial resistance (TEER) exceeded 300 Ω cm^2.

Transport experiments

Incubations were done in Hanks' buffer with 25 mM HEPES at 37 °C. Resveratrol (5–40 μM) was added to either the apical or the basolateral chamber after two 30-min buffer washes of the cell layers. Samples were withdrawn at specified times from both chambers for analysis by reversed-phase HPLC. [^{14}C]Mannitol, a marker of paracellular transport, was added to the apical chamber in each experiment. An apparent permeability coefficient (P_{app}) (Artursson 1990) of $< 0.5 \times 10^{-6}$ cm s^{-1} was considered acceptable.

HPLC analysis

Resveratrol samples were analysed on a Waters (Milford, MA) HPLC system consisting of a Model 717 Plus autosampler, a Model 510 pump and a Model 996 photodiode array detector with a Millennium software system. A Symmetry C18, 3.9 × 150 mm, column (Waters) was used with a mobile phase of methanol–glacial acetic acid–water (35:1:64) at a flow rate of 0.9 mL min^{-1} and detection at 305 nm. The specificity of this detection method was very high and the sensitivity even for the lowest concentration produced signal-to-noise ratios greater than 10. The reproducibility afforded coefficients of variation of less than 10%.

Resveratrol metabolism

Caco-2 cells (ca 2 weeks old) grown in 6-wells were incubated with resveratrol (100 μM) in complete cell culture medium. After 6 h, 1-mL samples of the medium were processed by solid-phase extraction. The samples were added to conditioned Oasis HLB cartridges (Waters) and washed with 5% methanol. Resveratrol and metabolites were eluted with 2 mL methanol, evaporated to dryness under nitrogen and reconstituted in 35% methanol before HPLC analysis.

To facilitate the identification of possible sulfate conjugates of resveratrol, [^{35}S]-labelled sodium sulfate was added to the medium in some incubations (Walle et al 1993). After the standard solid-phase extraction and counting a portion of the reconstituted samples, the samples were analysed by HPLC with 0.5-min fraction collection. ScintiSafe Econo 2 fluid (Fisher Scientific, Fair Lawn, NJ) was added and the fractions were subjected to liquid scintillation spectrometry.

The presumed sulfate conjugate was isolated by HPLC, taken to dryness and re-dissolved in 50 mM Tris buffer, pH 7.0. To half of the sample, 0.05 U of aryl sulfatase was added and both samples were incubated in a 37 °C water bath. After 2 and 30 min, portions of both samples were analysed by HPLC.

The presumed resveratrol glucuronide was also isolated by HPLC, taken to dryness and redissolved in phosphate buffer, pH 5. Beef liver β-glucuronidase (Sigma-Aldrich) was added to half of each sample. After one hour at 37 °C, the samples were analysed by HPLC (Galijatovic et al 1999).

HPLC/MS

HPLC/MS analyses used a Finnigan LCQ ion-trap mass spectrometer with electrospray ionization operated in the positive ion mode, similar to a previous report (Galijatovic et al 1999).

Cellular uptake of resveratrol

Caco-2 cells grown in 6-well plates for 27 days were incubated with 10 μM resveratrol in Hanks' buffer (pH 7.4) for 1 h. The buffer was collected for analysis and the cell monolayers were washed twice. The cells were then scraped off and pelleted and extracted 3 times with 1 mL of methanol. The combined methanol extracts were evaporated to dryness and reconstituted in mobile phase. Buffer and cell extracts were analysed by HPLC as described above. The volume of the packed cells was estimated to be able to

express cellular content of resveratrol in concentration terms (i.e. pmol mL^{-1}). The cell uptake/incubation buffer concentration ratio was then calculated.

Statistics

The statistical significance of differences between treatments was evaluated by using the two-tailed paired Student's *t*-test with a significance level of $P < 0.05$.

Results

In these cell culture experiments we first determined the apical-to-basolateral transport of resveratrol over 6 h, using a concentration range of 5–40 μM. This concentration is somewhat higher than would be anticipated in the intestinal lumen after drinking a glass of red wine (Sato et al 1997; Frémont 2000) but lower than would be anticipated after ingesting a 10–20 mg dose of resveratrol as a dietary supplement, taking into account gastric fluid volumes (Walgren et al 1998). Transcellular absorption clearly occurred (Figure 2), for all concentrations. However, the transport was linear for only about one hour. Also, the absorption appeared to be concentration dependent. This is better seen in Table 1, where the P_{app} for 1-h transport has been summarized. The corresponding P_{app} values for the paracellular transport marker mannitol, in the same experiments, were 0.41 ± 0.04 (mean $\pm$ s.e.m., n = 12). Thus, the apical to basolateral transport rate more than doubled when the resveratrol concentrations increased from 5 to 40 μM. When basolateral-to-apical efflux was determined, the values at the lower concentrations were virtually identical to the absorption (Table 1), demonstrating the absence of direction-dependent transport. However, for the basolateral-to-apical transport there was no concentration dependence.

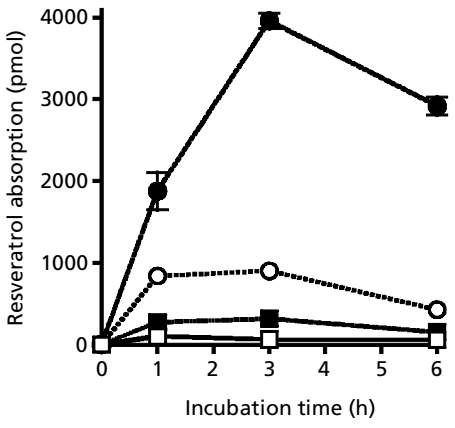

Figure 2 Apical-to-basolateral absorption of resveratrol in Caco-2 cells. □, 5 μM; ■, 10 μM; ○, 20 μM; ●, 40 μM. Mean values $\pm$ s.e.m., n = 3, for each time point shown. For most time points the s.e.m. was smaller than the symbol used.

Table 1 Transport rates for different concentrations of resveratrol in Caco-2 cells.

Resveratrol concn (μM)	Apical-to-basolateral transport (P_{app})	Basolateral-to-apical transport (P_{app})
5	5.6 ± 1.6	7.8 ± 0.5
10	7.6 ± 0.5	6.7 ± 0.6
20	11.6 ± 1.0	7.2 ± 0.3
40	13.0 ± 1.6*	8.2 ± 1.8

P_{app} is expressed in cm s^{-1} ($\times 10^{-6}$); values are means $\pm$ s.e.m., n = 3.
*$P < 0.05$, vs the value for 5 μM.

The limited linearity in transport with time could suggest extensive metabolism of resveratrol by the Caco-2 cells. Its metabolism was investigated by incubating a high concentration (100 μM) of resveratrol with Caco-2 cells cultured in 6-well plates for 6 h to facilitate identification of metabolites. Extraction of resveratrol and its potential metabolites by a solid-phase procedure and HPLC analysis produced the tracing in Figure 3. The highly selective wavelength of 305 nm for resveratrol permitted easy detection of two metabolites, I and II, which were not present in the absence of resveratrol. When the sample was incubated with β-glucuronidase, peak I disappeared quantitatively and the resveratrol peak increased, indicating that peak I was a resveratrol glucuronide. This was confirmed by HPLC/MS, demonstrating an $(M + H)^+$ ion of m/z 405 with subsequent loss of 176 a.m.u. (glucuronic acid moiety). Similarly, when the sample was incubated with sulfatase, peak II disappeared, with a concomitant increase in the resveratrol peak. Confirmation of peak II as a sulfate conjugate by HPLC/MS failed. A previous study clearly showed great difficulties obtaining mass spectral data for polyphenol sulfate conjugates (Galijatovic et al 1999). However, when the incubations were performed in the presence of radioactive inorganic sulfate ($Na_2{}^{35}SO_4$) (Walle et al 1993), peak II became radiolabelled, indicating that it was a sulfate conjugate. The broad, tailing peak II is also highly characteristic of many polyphenol sulfate conjugates, such as that of chrysin sulfate (Galijatovic et al 1999).

We next examined the major sulfate conjugate (peak II) under transport conditions. Shown in Figure 4 is the appearance of this conjugate on both the apical and basolateral sides after loading the lower 5–40 μM resveratrol concentration on the apical side. At 5 μM resveratrol, most of the resveratrol sulfate is effluxed to the apical side with only small amounts appearing on the basolateral side. As the resveratrol concentration increases, there is an increasing amount of the sulfate conjugate on the basolateral side, with no change in the amount on the apical side, thus, resulting in a shift of the cellular export in favour of the basolateral side. However, at 40 μM resveratrol, there was an abrupt decrease in the export of the sulfate towards both sides. The amount of the glucuronic acid conjugate formed (peak I) was very low in comparison (i.e. less than 5% of total conjugates) and all of it was exported to the

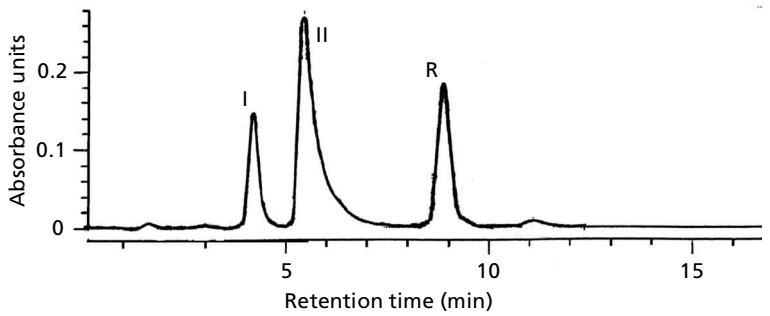

Figure 3 HPLC tracing of cell culture medium harvested after incubating Caco-2 cells with 100 μM resveratrol for 6 h. Resveratrol (R) and metabolites (I and II) were first isolated from the medium by solid-phase extraction. Detection was by UV at 305 nm.

apical side. Similar analysis of metabolites was done after loading resveratrol on the basolateral side. The results were virtually identical to those obtained after apical loading of resveratrol.

As resveratrol was efficiently transported through the Caco-2 cell monolayer as well as metabolized by sulfate conjugation, we were also interested in whether resveratrol accumulated in the cells. Caco-2 cells grown in 6-well plates were incubated with 10 μM resveratrol in Hanks' buffer for 1 h. The cells were rapidly washed, scraped off the plates, extracted with methanol and the packed cell volume estimated. A very high cell/buffer resveratrol concentration ratio of 38.5 ± 2.5 ($n = 4$) was found.

Discussion

This study, using the human intestinal cell line Caco-2 cultured as a monolayer on permeable membranes, indicates that resveratrol easily traverses the enterocyte and therefore likely will be absorbed in-vivo. However, the oral bioavailability may be limited, as the enterocyte efficiently metabolizes resveratrol, mainly by sulfate conjugation and to a lesser extent by glucuronidation.

Resveratrol appears to be subject to transcellular absorption with no direction dependency in its transport. The P_{app} value of $\approx 7 \times 10^{-6}$ cm s^{-1} at 1 h would predict a high absorption in-vivo (Artursson & Karlsson 1991; Yee 1997). This is consistent with the high degree of absorption that has been suggested in the rat (Andlauer et al 2000; Soleas et al 2001; Juan et al 2002). The transport was linear for only about one hour, similar to previous observations for another polyphenol, chrysin (Galijatovic et al 1999). At longer times there was a reduction in transport, which was clearly concentration dependent. This may be associated with an increased fraction of resveratrol being metabolized. When attempting to extrapolate these data to the in-vivo situation, this may indicate a very low oral bioavailability, as seen when extrapolating similar data for the flavonoid chrysin from the in-vitro (Walle et al 1999) to the in-vivo (Walle et al 2001) situation.

The transport rate of resveratrol more than doubled at 40 μM (Table 1). At this concentration, the export of the sulfate conjugate was dramatically reduced (Figure 4).

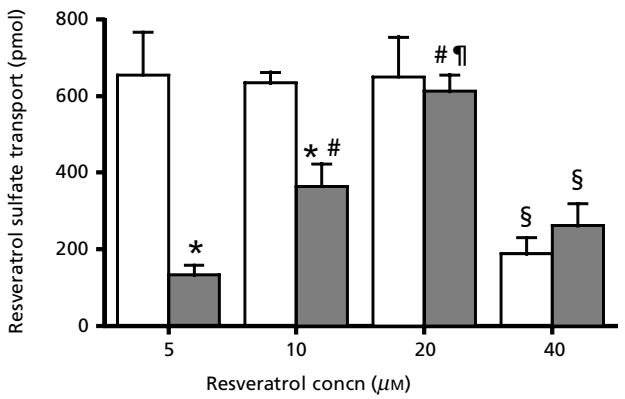

Figure 4 Appearance of resveratrol sulfate (peak II in Figure 3) on the apical (open bars) vs basolateral (filled bars) sides after loading resveratrol on the apical side. The observations were made after 1-h incubations. Mean values ± s.e.m., $n = 3$, for each concentration are shown. *$P < 0.05$, vs apical side; $P < 0.05$, vs 5 μM; ¶$P < 0.05$, vs 10 μM; §$P < 0.05$, vs 20 μM.

The mechanism of this effect is not clear but may be due to opening of the tight junctions, letting resveratrol cross the monolayer between the cells rather than through the cells.

Although the metabolism of resveratrol has been extensively studied, it is not clear which pathway(s) predominate in man. In the rat, whether involving in-vivo studies (Kuhnle et al 2000; Juan et al 2002) or using the perfused small intestine (Andlauer et al 2000; Kuhnle et al 2000), glucuronidation clearly predominates, with a small contribution by sulfation. In man, using in-vitro enzyme preparations (De Santi et al 2000a, b; Aumont et al 2001), both glucuronidation and sulfation may occur. In this study, we clearly demonstrate in the enterocyte that sulfate conjugation is more important than glucuronidation, particularly during transport conditions with physiologically relevant concentrations of resveratrol. Thus, a major species difference seems to exist. When we employed a higher concentration (100 μM) to identify the metabolites, glucuronidation became more significant (Figure 3), probably due to inhibition of sulfate conjugation.

In the cell uptake experiments, we only observed resveratrol, not its abundant sulfate conjugate. This emphasizes the efficient export of this conjugate. At the lowest resveratrol concentration, the sulfate conjugate mainly appeared on the apical side (Figure 4). This also occurred with the small amount of glucuronic acid conjugate formed. This is similar to previous findings with the flavonoid chrysin (Walle et al 1999) and is likely due to the apically localized MRP2 transporter (Walle et al 1999; Walgren et al 2000). However, as the resveratrol concentrations increased, there was a shift in the sulfate conjugate export towards the basolateral side with no change in the apical export. This suggests saturation of MRP2 export, forcing the sulfate conjugate towards the basolateral side, maybe via basolaterally localized MRP3 (Bock-Hennig et al 2002). Thus, potential interaction between resveratrol intake and MRP transporters is suggested. This should be examined in more detail as interaction with drugs, carcinogens and other nutrients may be anticipated.

Finally, the finding of an almost 40-fold accumulation of resveratrol in the Caco-2 cells in comparison with the incubation buffer is remarkable and emphasizes that the enterocyte could be a major biological target site for this dietary preventive compound.

Conclusion

The transcellular transport of resveratrol by the human in-vitro absorption model Caco-2 is high and independent of direction, suggesting facile intestinal absorption in-vivo in man. However, limited linearity in the transport rate with time and extensive metabolism suggest that extensive pre-systemic metabolism of resveratrol may occur, leading to low oral bioavailability. Metabolic studies demonstrate that sulfate conjugation is the major pathway for resveratrol in the Caco-2 cells and probably also in man in-vivo. Highly polarized export of the sulfate conjugate from the Caco-2 cells occurred, which was concentration dependent. In addition, resveratrol showed very high, 35-fold, accumulation in the Caco-2 cells, which may be critical for its biological effects.

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