Transport mechanism and metabolism of olive oil hydroxytyrosol in Caco-2 cells

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Abstract 3,4-Dihydroxyphenylethanol (hydroxytyrosol; DPE) is the major phenolic antioxidant present in extra virgin olive oil, either in a free or esterified form. Despite its relevant biological effects, no data are available on its bioavailability and metabolism. The aim of the present study is to examine the molecular mechanism of DPE intestinal transport, using differentiated Caco-2 cell monolayers as the model system. The kinetic data demonstrate that [^{14}C]DPE transport occurs via a passive diffusion mechanism and is bidirectional; the calculated apparent permeability coefficient indicates that the molecule is quantitatively absorbed at the intestinal level. The only labelled DPE metabolite detectable in the culture medium by HPLC (10% conversion) is 3-hydroxy-4-methoxyphenylethanol, the product of catechol-*O*-methyltransferase; when DPE is assayed in vitro with the purified enzyme a K_m value of 40 μM has been calculated.

Key words: Antioxidant; Hydroxytyrosol; Olive oil; Polyphenol; Transport; Caco-2 cell

1. Introduction

Polyphenols are dietary antioxidants, widely distributed in the vegetable kingdom [1], which exert a variety of biochemical and pharmacological roles, including antiinflammatory [2], antithrombotic [3] and antineoplastic [4,5] activities.

Among these compounds, great attention has been paid to hydroxytyrosol (3,4-dihydroxyphenylethanol; DPE), a simple phenolic compound, endowed with powerful free radical scavenging activity [6–8]. This liposoluble and hydrosoluble molecule is present in high concentration in extra virgin olive oil, either in a free or esterified form, as oleuropeine aglycone [9].

There is clear epidemiological evidence that virgin olive oil greatly contributes, together with fruit and vegetables, to the well known effect of the Mediterranean diet in lowering the incidence of several degenerative pathologies, including coronary heart diseases [10,11] and cancer [12–14]. In this respect, converging evidence indicates that olive oil's beneficial effects are related not only to the elevated oleic acid content but also to the high level of vitamin and non-vitamin antioxidants, including DPE [15–17].

The biological activities of DPE have been explored by several groups and recently reviewed in [16]. DPE prevents LDL oxidation [15], platelet aggregation [18] and inhibits 5- and 12-lipoxygenases [19,20]. It also exerts an inhibitory effect on peroxynitrite dependent DNA base modification and tyrosine nitration [21]. Moreover, data from our laboratory have demonstrated that DPE counteracts cytotoxicity induced by reactive oxygen species (ROS) in various human cellular systems, such Caco-2 cells [22] and erythrocytes [23]. Finally, we recently reported that DPE induces apoptosis in HL-60 cells [16].

Despite the variety of DPE biological effects, so far no data have been reported on its bioavailability and metabolism, with the single exception of a preliminary in vivo study on DPE intestinal absorption in rats [24]; however, the molecular mechanism of its uptake was not investigated.

The aim of this paper is the elucidation of the kinetics of [^{14}C]DPE intestinal transport and its metabolism, using differentiated Caco-2 cell monolayers as the model system of the human intestinal epithelium. As a matter of fact, these cells, which closely mimic in vitro the food-intestinal tract interaction [25], have been successfully utilized to demonstrate the DPE protective effect against ROS-mediated cytotoxicity [22].

2. Materials and methods

2.1. Materials

[^{14}C]DPE (2.25 mCi/mmol) was custom synthesized by NEN Life Science Products (Cologno Monzese, Italy). D-[2-^{3}H]Glucose (20 Ci/mmol) and D-[^{14}C]mannitol (57 mCi/mmol) were obtained from Amersham International (Little Chalfont, UK).

Cell culture media, serum and reagents were purchased from GIBCO BRL. Multiwells (BIO COAT collagen I cellware) were provided by Labtek srl (Milan, Italy), and Transwell polycarbonate microporous cell culture (3.0 mm pore size; 1 cm^2 surface area) by Corning Costar (Cambridge, MA, USA).

All the other chemicals used in this study, including DPE analogs, were from Sigma-Aldrich (St. Louis, MO, USA).

2.2. Cell culture

The Caco-2 cell line was kindly provided by Dr. Leone (University of Salerno, Italy) and used between passages 80–90. The cells were routinely maintained at 37°C in a 5% CO_2 atmosphere in high glucose DMEM, supplemented with 10% FCS and antibiotics (5×10^4 IU/l penicillin, 50 mg/l streptomycin).

2.3. Cell lysis buffer

The cell lysis buffer consists of: 20 mM Tris–HCl, 500 mM NaCl, 0.5 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl-fluoride, 0.25% Triton X-100, pH 7.4.

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Abbreviations: DPE, hydroxytyrosol (3,4-dihydroxyphenylethanol); HMPE, homovanillic alcohol (3-hydroxy-4-methoxy phenylethanol); PE, tyrosol (4-hydroxyphenylethanol); PC, pyrocatechol (1,2-dihydroxybenzene); L-DOPA, 3,4-dihydroxy phenylalanine; COMT, catechol-*O*-methyltransferase; P_{app}, apparent permeability; ROS, reactive oxygen species

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2.4. Determination of the kinetics of DPE transport in Caco-2 cells

For kinetics experiments Caco-2 cells were seeded at a density of 90 000 cells/cm^2 in collagen-treated multiwell dishes, and the medium was changed every 48 h. 12–14 days after confluence, differentiated monolayers were washed once with PBS and then incubated for 2 min in PBS containing increasing amounts of [^{14}C]DPE. At the end of the incubation, the medium was rapidly removed and the cells were washed once with ice-cold PBS, containing 1 mM unlabelled DPE. Finally, cells were lysed with 500 μl of lysis buffer and the amount of [^{14}C]DPE taken up by Caco-2 was measured by liquid scintillation counting.

2.5. Evaluation of transepithelial transport in Caco-2 cells

For transepithelial transport experiments Caco-2 cells were seeded at a density of 90 000 cells/cm^2 in the insert of a transwell culture chamber, and the medium (0.2 ml in the insert and 0.8 ml in the well) was changed every 48 h. 12–14 days after confluence, the integrity of the monolayer of differentiated cells was monitored by measuring the transepithelial electrical resistance value, according to Hidalgo et al. [25]; only samples with values $>300\ \Omega\ cm^2$ were utilized. For apical to basolateral flux measurements, the medium of the insert was removed and replaced with 0.2 ml of PBS, containing either [^{14}C]mannitol (1 μM) or [^{3}H]glucose (100 μM) or [^{14}C]DPE (100 μM). At different time intervals, the radioactivity of the medium of the receiving compartment (the well) was measured. The apparent permeability (P_{app}) coefficients were calculated as in [26].

2.6. HPLC analysis

To investigate the metabolic transformations of DPE following transepithelial transport, Caco-2 cells were incubated as described in Section 2.5 and the media of the receiving compartment were analyzed by reversed-phase chromatography, using a gradient of 0.2% acetic acid in water, pH 3.1/methanol as the mobile phase, as described by Montedoro et al. [27] for the analysis of olive oil polyphenols.

2.7. Catechol-O-methyltransferase (COMT) assay

To test DPE as substrate for COMT (EC 2.1.1.6.) activity, [^{14}C]DPE (50 μM) was incubated in the presence of 30 units of purified enzyme (porcine liver, from Sigma) and a saturating concentration (100 μM) of the methyl donor *S*-adenosyl-methionine. The assay mixture also contained 1 mM $MgCl_2$ and 400 mM Tris–HCl, pH 8.0. At the end of 1 h incubation at 37°C, the reaction was stopped by the addition of 1 volume of 1 N HCl, and aliquots of the mixture were analyzed by HPLC as reported in Section 2.6. For kinetic analysis COMT activity was assayed as reported in [28].

2.8. Protein concentration

Protein concentration was estimated according to the Bradford's method [29], using a Bio-Rad protein assay kit I.

2.9. Statistical analysis

Results are expressed as means ± S.E.M. of three or more determinations. Differences in P_{app} coefficients were evaluated using Student's *t*-test and were considered significant at $P<0.05$.

3. Results and discussion

3.1. Kinetics of DPE transport in Caco-2 cells

The kinetics of DPE intestinal transport was evaluated in differentiated Caco-2 cells, grown on collagen-treated multiwells, 12–14 days post confluence. In these experimental conditions Caco-2 cells undergo spontaneous differentiation to form a polarized monolayer of cells which express morphological and functional characteristics of normal enterocytes [25].

Uptake was followed at 37 and 4°C in the presence of increasing amounts of [^{14}C]DPE. At the end of the 2 min incubation, incorporated [^{14}C]DPE was monitored as described in Section 2. The results show that the rate of DPE uptake is linear in the 50–500 μM range, both at 37 and at 4°C (Fig. 1), indicating that the intestinal transport system is not saturable. Moreover, to further confirm that transport occurs via a passive diffusion mechanism, the time course of DPE efflux from Caco-2 cells was analyzed. Cells, preloaded with [^{14}C]DPE, were incubated in culture medium for different time intervals and the residual radioactivity present in the cells was monitored. The results (Fig. 2), show that only 28% of incorporated [^{14}C]DPE is still present within the cells after a 60 s incubation, indicating a rapid back diffusion of the molecule.

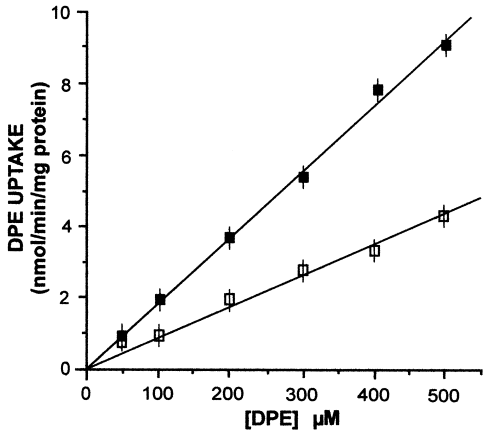


Fig. 1. Concentration dependence of DPE transport in Caco-2 cells. DPE uptake was measured at 37 (■) and 4°C (□) after 2 min incubation, in the presence of increasing concentrations of [^{14}C]DPE. Values are expressed as means ± S.E.M. of three experiments.

The uptake of [^{14}C]DPE was also measured in the presence of structurally related phenols, assayed as potential competitive inhibitors of DPE transport: a 10 fold excess of pyrocatechol (1,2-dihydroxybenzene; PC), tyrosol (4-hydroxyphenylethanol; PE) or homovanillic alcohol (3-hydroxy-4-methoxy phenylethanol; HMPE) does not reduce DPE uptake. Similarly, 3,4-dihydroxy phenylalanine (L-DOPA), assayed at a concentration saturating its carrier protein [30], does not influence the rate of DPE uptake (Fig. 3).

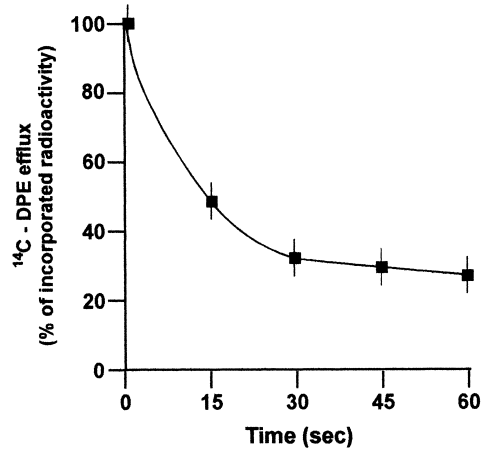
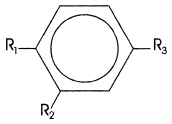


Fig. 2. Time course of [^{14}C]DPE efflux from loaded Caco-2 cells. Cells were preincubated with 500 μM [^{14}C]DPE, washed once with ice-cold PBS and then incubated in PBS at 37°C for different time intervals. At the end of incubation, residual radioactivity present in the cells was measured. Values are expressed as means ± S.E.M. of three experiments.



ANALOG	R_1	R_2	R_3	ANALOG/DPE RATIO	DPE UPTAKE (nmol/min/mg protein)
DPE	OH	OH	CH_2-CH_2OH	-	1.9 ± 0.13
HMPE	OH	OCH_3	CH_2-CH_2OH	10	1.8 ± 0.15
PE	OH	H	CH_2-CH_2OH	10	2.0 ± 0.18
PC	OH	OH	H	10	1.9 ± 0.16
L-DOPA	OH	OH	$CH_2CH(NH_2)COOH$	5	1.8 ± 0.10

Fig. 3. Structurally related phenols assayed as potential competitive inhibitors of DPE transport in Caco-2 cells. The experimental conditions of DPE uptake are reported in Section 2.

3.2. Transepithelial DPE transport through a monolayer of Caco-2 cells

In order to evaluate DPE transepithelial transport, experiments were performed using Caco-2 cells cultured on a transwell cell culture chamber. In this system, indeed, cells grow and differentiate on a polycarbonate membrane, separating two different compartments, the basal and the apical, towards which the luminal part of the polarized epithelium is oriented.

When [^{14}C]DPE is added to the apical compartment, a significant transepithelial DPE flux (Ap→BL) is observable. After 1 h incubation, as much as 25% of the applied radioactivity is recovered in the basal compartment (data not shown). Similarly, a time-dependent transport occurs when [^{14}C]DPE is applied from the basolateral side of the monolayer (BL→Ap).

The data on DPE transepithelial transport were utilized to calculate the P_{app} (P_{app}) coefficients [26]; as markers of active and paracellular transport, the P_{app} for glucose and mannitol were also measured. As shown in Table 1, the P_{app} value for the Ap→BL DPE transport is similar to that in the BL→Ap direction, indicating that DPE intestinal transport is bidirectional. These values are of the same order of magnitude as the P_{app} for glucose; as expected, no transport is observable for mannitol. Moreover, the P_{app} value calculated for Ap→BL transport ($12.4 \pm 0.9 \times 10^{-6}$ cm s^{-1}) suggests that DPE is quantitatively absorbed following its oral administration. A previous study, indeed, positively correlates P_{app} coefficients, obtained in vitro on Caco-2 cells, with the absorption of drugs and nutrients in humans [26], indicating 100% absorption for P_{app} values $> 10^{-6}$.

3.3. DPE metabolism in Caco-2 cells

In order to investigate the metabolism of DPE in human enterocytes, the media of the receiving compartments were analyzed by HPLC, at the end of transepithelial transport. The results, reported in Fig. 4a, show that, after 1 h incubation, DPE still represents the major molecular species, accounting for about 90% of the total radioactivity of the medium. The only labelled DPE metabolite is its methylated derivative HMPE, a product of intestinal COMT activity. When DPE was assayed in vitro in the presence of the methyl donor *S*-adenosylmethionine and purified COMT from porcine liver a K_m of 40 μM was calculated (Fig. 4b). This value is significantly lower than that of the endogenous substrates, including dopamine [31–33], suggesting that DPE could be a preferential substrate for this enzyme in vivo.

In conclusion, the reported data provide the first experimental evidence of the high bioavailability of DPE: this finding strengthens the hypothesis that the nutritional benefits of olive oil can be related to the high content of DPE and its precursor, oleuropeine aglycone. Finally, these data, together with the well documented antioxidant, antithrombotic and antiproliferative effects of DPE, make this molecule a good candidate for the chemoprevention of cancer and vascular diseases.

Table 1
P_{app} coefficients for DPE transepithelial in Caco-2 cells

Compound	Transport direction	P_{app} ($\times 10^{-6}$ cm s^{-1})
DPE	Ap→BL	12.4 ± 0.9
DPE	BL→Ap	13.7 ± 1.1
Mannitol	Ap→BL	0.32 ± 0.02
Glucose	Ap→BL	23.4 ± 1.8

The experimental conditions are reported in Section 2. Data are expressed as means ± S.E.M. of six determinations. Ap→BL indicates apical to basolateral transport. BL→Ap indicates basolateral to apical transport.

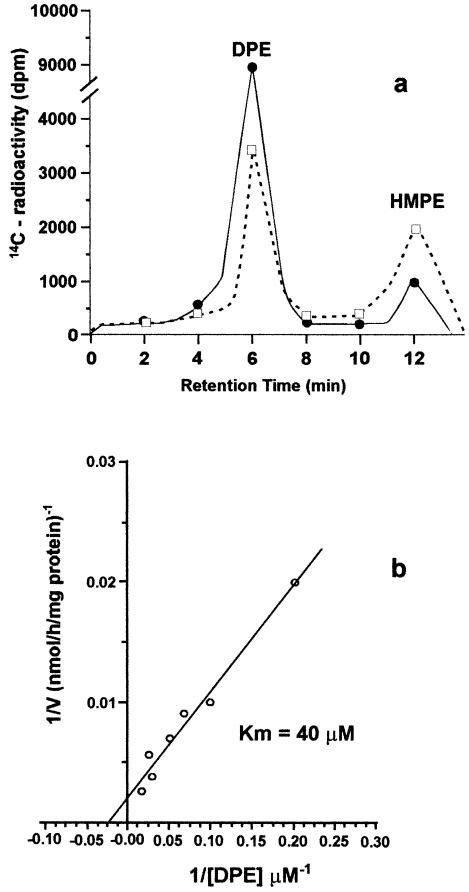


Fig. 4. a: HPLC separation of [^{14}C]DPE and its metabolites. solid: Analysis of cell culture medium following Ap→BL DPE transepithelial transport in Caco-2 cells; dashed: Analysis of the assay mixture for COMT activity, using [^{14}C]DPE as substrate. b: Lineweaver–Burk plot of COMT activity as a function of increasing DPE concentrations. The experimental conditions are reported in Section 2.

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