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ORIGINAL CONTRIBUTION

Mathieu Silberberg
Christine Morand
Thierry Mathevon
Catherine Besson
Claudine Manach
Augustin Scalbert
Christian Remesy

The bioavailability of polyphenols is highly governed by the capacity of the intestine and of the liver to secrete conjugated metabolites

Summary *Background* After ingestion of a complex meal containing foods and beverages of plant origin, different polyphenols are likely to be simultaneously present in the intestine. However, almost nothing is known about their interactions and possible consequences on their bioavailability. *Aim of the study* The present study deals with the intestinal absorption and splanchnic metabolism of three polyphenols, genistein, hesperetin and ferulic acid (FA), when perfused in the small intestine alone or in combination, at different doses (15 and 120 μM). *Methods* The fate of polyphenols in the small intestine was studied using a rat *in situ* intestinal perfusion model. Polyphenols were analysed in perfusate, bile and plasma by HPLC. *Results* Whatever the perfused dose, the efficiency of the net transfer towards the enterocyte was similar for the three polyphenols and not significantly modified by any association between these molecules. However, FA largely differed from the two flavonoids by its low intestinal secretion of conjugates. When perfused at 15 μM, the secretion of conjugates back to the lumen represented 6.2 % of the net transfer into the enterocytes for FA compared to 25.5 and 20 % for genistein and hesperetin respectively. Intestinal conjugation and secretion of conjugates back to the gut lumen varied with the dose of flavonoids: saturation of conjugation was observed for the highest dose or when a high dose of a second flavonoid was perfused simultaneously. Intensity of the biliary secretion substantially differed among tested polyphenols: 7.7 % of the net transfer for FA vs 50 % for genistein or hesperetin. The extent of the enterohepatic cycling of these polyphenols was proportional to the perfused dose and unaffected by the simultaneous presence of different compounds in the intestine. *Conclusion* Genistein and hesperetin appeared less available than FA for peripheral tissues because of a high intestinal and biliary secretion of their conjugates. Moreover, data suggest that a high polyphenol intake may improve their bioavailability due to saturation of the intestinal secretion of conjugates.

Key words rats – polyphenols – in situ perfusion – intestinal metabolism – biliary secretion

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M. Silberberg · C. Morand (✉) · C. Besson · C. Manach · A. Scalbert · C. Remesy
Laboratoire des Maladies Métaboliques et des Micronutriments
I. N. R. A. Centre de Recherche de Clermont-Ferrand/Theix
63122 Saint Genès-Champanelle, France
Tel.: +33-47/362-4681
Fax: +33-47/362-4638
E-Mail: cmorand@clermont.inra.fr

T. Mathevon
Centre Hospitalier Universitaire de Clermont-Ferrand
Service Accueil Urgences
Clermont-Ferrand, France

Introduction

Polyphenols refer to one of the numerous and widely distributed groups of molecules in the plant kingdom. Based on their structure they are classified into different families, phenolic acids, flavonoids, or less common lignans and stilbenes. Polyphenols have gained increasing interest because of their numerous biological effects such as free-radical scavenging, modulation of enzymatic activities, and inhibition of cellular proliferation, as well as their potential utility as antibiotic, anti-allergic and anti-inflammatory agents [1]. They may participate in the prevention of cardiovascular diseases, cancers and other degenerative diseases [2]. Because they are widely distributed in foods and beverages of plant

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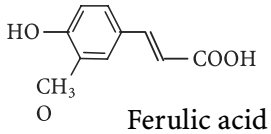
origin (such as fruit, vegetables, cereals, tea, coffee, cocoa, wine and fruit juice), polyphenols are common micronutrients in human diet. Total polyphenol intake may commonly reach 1 g/d. This daily intake is mainly constituted by hydroxycinnamates and flavonoids, which account respectively for about 1/3 and 2/3 of the total intake [3].

The physiological interest of dietary polyphenols depends on their intestinal absorption and their subsequent interactions with target tissues; however the mechanisms of gastrointestinal absorption of polyphenols are not completely understood. Polyphenols linked to a rhamnose moiety have to reach the colon to be hydrolysed by the microflora prior to their absorption, whereas aglycones and some glucosides are absorbed in the small intestine [4, 5]. One relevant approach to study the intestinal absorption and metabolism of polyphenols consists to perform *in situ* intestinal perfusion of the rat small intestine. Using this technique, intestinal and biliary parameters that largely govern the bioavailability of polyphenols are measured: efficiency of the net transfer through the brush border, intestinal metabolism and secretion of conjugates, rate of biliary secretion. This model, developed a few years ago in our lab, has been used to characterize the intestinal absorption and splanchnic metabolism of various isolated flavonoids [6].

Consumption of plant foods is related to the simultaneous intake of numerous dietary polyphenols, each of them present at different concentrations. Thus interactions between these compounds in the intestine may influence their final respective bioavailability. To study these possible interactions, we have followed the fate in the splanchnic area of some representative members of two main families of polyphenols which largely differed by their structure, namely one hydroxycinnamate and two flavonoids. Ferulic acid (FA) is a hydroxycinnamate, especially abundant in cereal brans [7]. Consuming high quantities of cereal products can provide more than 100 mg of ferulic acid per day. Flavanones and isoflavones are restricted to a few foodstuffs. However, they are important dietary sources of flavonoids since their content reaches 500 mg/l in orange juice and 1 g/kg in soya bean, respectively [8, 9]. Numerous studies have reported the biological effects of isoflavones and relevance to human health [10, 11]. Studies demonstrating protective effects of flavanones on human health are still limited [12]; however, in animal studies flavanones exhibit interesting effects on parameters involved in cardiovascular diseases [13, 14] and protect against chemically induced cancers [15–17].

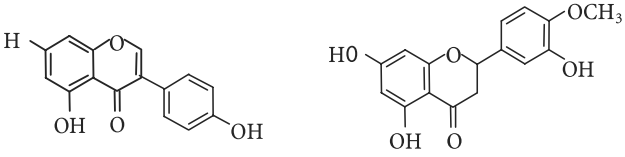
FA, genistein and hesperetin (Fig. 1) were used in *in situ* intestinal perfusion experiments to investigate the influence of the structure, the dose and possible interactions between polyphenols in the intestine, on their respective fate in the splanchnic area.

Hydroxycinnamic acid:



Ferulic acid

Flavonoids:



Genistein Hesperetin

Fig. 1 Chemical structures of perfused polyphenols

Materials and methods

Chemicals

FA, genistein and hesperetin were purchased from Extrasynthese (Genay, France). β-glucuronidase/sulfatase from Helix Pomatia, liver UDP-glucuronosyl transferase (UDP-GT), UDP-glucuronic acid and UDP-N-acetyl glucosmamine (UGP-NAG) were purchased from Sigma (L'Isle D'Abeau, Chesnes, France)

Animals and diets

Before perfusion experiments, male Wistar rats (about 150 g) were housed two per cage in temperature-controlled rooms (22 °C) with a dark period from 20:00 to 8:00 h and had access to food from 16:00 to 8:00 h. They received a standard semi-purified diet (73 % wheat starch, 15 % casein, 6 % mineral mixture (AIN 93M), 1 % vitamin mixture (93VX), and 5 % corn oil) for 2 weeks. Animals were maintained and handled according to the recommendations of the Institut National de la Recherche Agronomique Ethics Committee, as legally required.

Sampling procedure

For the study 24-h fasted rats were anesthetized with sodium pentobarbital by intraperitoneal administration (40 mg/kg body weight) and maintained alive on a heating pad throughout the perfusion period. The biliary duct was first cannulated, then cannulas were introduced at the extremities of the jejunal + ileal segment

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(from 5 cm distal from the flexura duodenojejununalis to the valvula ileocoecalis). This segment was continuously perfused *in situ* for 45 min without recycling, with a physiological and thermostated (37 °C) buffer pH 6.6 supplemented with the selected polyphenol(s), just before use. The composition of the basal perfused buffer was as followed: KH_2PO_4 (5 mM), K_2HPO_4 (2.5 mM), $NaHCO_3$ (5 mM), NaCl (50mM), KCl (40 mM), tri-potassium citrate (10 mM), $CaCl_2$ (2 mM), $MgCl_2$ (2 mM), glucose (8 mM), and taurocholic acid (1 mM) at pH 6.6. Just before the perfusion, the addition of the selected polyphenols to the buffer was performed from a concentrated solution in DMSO (DMSO in the final perfusion buffer never exceeded 0.5 ‰). The flow rate was set at 0.8 mL/min and FA, genistein and hesperetin were perfused at 15 µM or 120 µM leading to perfused fluxes of respectively 12 and 96 nmol/min. (Note: the perfused fluxes presented in the Table 1 may slightly differ from these theoretical values (±10 %) due to the exact determination of the concentration of polyphenols in the perfusion buffer for each experiment). The effluent at the exit of the ileum and the bile were collected throughout the perfusion and stored at –20 °C until analysis.

At the end of the experiment, blood samples were withdrawn from the mesenteric vein and from the abdominal aorta and sampled into heparinized tubes. Immediately after their sampling, plasma samples were acidified with acetic acid (10 mM final) and stored at –20 °C until analysis.

HPLC analysis

Sample treatment

Bile, plasma and perfusate samples were acidified (to pH 4.9) with 0.1 volume of 0.58 M acetic acid and incubated for 30 min at 37 °C with (for total forms) or without (for unconjugated forms) $5x10^6$ units/L β-glucuronidase and $2.5x10^5$ units/L sulfatase. After addition of 2.85 vol. of methanol/200 mM HCl and centrifugation at 14,000 × g for 4 min, the resulting supernatants were analysed by HPLC-UV as described below. Calibration curves were prepared in the perfusion buffer, plasma or bile, by spiking control pools with known concentrations of FA, genistein and hesperetin (from 5 to 100 µM in the perfusion buffer, from 1 to 10 µM in the plasma, from 2.5 to 25 µM in the bile). These standards were then treated exactly as the samples. Quality control samples (duplicates of two different concentrations) were added in each batch of analyses to control the accuracy of the quantification. Differences between the measured value and the actual value never exceeded 5 % for all batches.

Chromatographic conditions

The HPLC system consisted of an autosampler (Kontron 360), fitted with a 5 µm C-18 Hypersil BDS analytical column (150 x 4.6 mm; Life Sciences International, Cergy, France). Mobile phase A consisted of 15 % acetonitrile and mobile phase B consisted of 30 % acetonitrile, for each phase the water fraction contained 0.5 % of H_3PO_4. The flow rate was set at 1 mL/min and the gradient conditions were as follows: 0–5 min: 100 % A, 5–35 min: linear gradient from 100 % A to 100 % B, 35–40 min: 100 % B, 40–50 min: 100 % A. The UV-detector was set at 320 nm. Retention times for FA, genistein and hesperetin were 10.4, 35.0 and 38.8 min, respectively.

Determination of the intestinal and biliary fluxes

All the calculated fluxes were expressed in nmol/min. The fluxes in the effluent have been calculated by taking into account the intestinal absorption of water as previously described [18]. The net transfer in the enterocyte was measured by the difference between the perfused flux and the flux in the nonhydrolysed effluent at the end of the perfusion. The secretion of conjugates back in the lumen resulted from the difference between the fluxes measured in the hydrolysed and nonhydrolysed effluent. The net absorption was obtained by the difference between the perfused flux and the flux measured in the hydrolysed effluent. The biliary secretion of conjugates resulted from the product between the biliary flow rate (µL/mL) and the concentrations of the total forms present in the bile after enzymatic hydrolysis (µM).

Activity of purified UDP-GT towards different phenolic substrates

The reaction mixture (in a final volume of 750 µl) consisted of 15 or 120 µM of the polyphenolic substrate dissolved in DMSO, 10 mM $MgCl_2$, 4 mM UDP-glucuronic acid, 2 mM UDP-N-acetyl glucosamine and 0.2 mg protein (purified liver UDP-GT), in 25 mM Hepes, pH 7.4. This mixture was incubated during 60 min (linear phase) during which time linear rates were obtained at 37 °C. Then, aliquots of the reaction mixture were taken, polyphenols were extracted and analysed by HPLC as described above. The enzyme activity was expressed in nmol of glucuronidated substrates/min/mg protein.

Statistics

Values are means ± SEM, and the differences between values were determined by one-way ANOVA coupled with the Tukey-Kramer multiple comparisons test. Values of $P < 0.05$ were considered significant.

Results

Intestinal perfusion of single polyphenols

FA, genistein or hesperetin were separately perfused into the rat small intestine at a concentration of 15 µM, hence fluxes of approximately up to 12.5 nmol/min were obtained (Table 1). The rate of the net transfer of these three polyphenols to the intestinal wall was similar and particularly high: 9.3, 9.8 and 9.7 nmoles/min for respectively FA, genistein and hesperetin. Some conjugated metabolites were recovered in the effluent at the end of the perfusion period. These conjugates had an intestinal origin since the biliary duct was cannulated before starting the perfusion. The rate of intestinal secretion of conjugates was dependent on the perfused compound. As shown in Table 1, the intestinal secretion of genistein and hesperetin conjugates reached 2.5 and 2.0 nmoles/min respectively, whereas the secretion level in the lumen of FA conjugates was only 0.6 nmoles/min.

The rate of the biliary secretion differed substantially between FA and the two flavonoids tested (Table 1): it was 0.7 ± 0.1 nmoles/min for FA, whereas it reached 5.1 ± 0.2 and 4.8 ± 0.5 nmoles/min for genistein and hesperetin. This suggests that the enterohepatic cycling of the two selected flavonoids is more effective than for FA.

To look for differences in intestinal and hepatic metabolism, we have determined, for each compound, the fractions of plasma metabolites present as conjugates or as aglycones in the mesenteric vein and in the abdominal aorta (Fig. 2). Because the absorbed metabolites are directly delivered into the mesenteric vein, the proportion of conjugates found in this vascular area is a good reflection of intestinal conjugation. For genistein and hesperetin more than 95 % of the total metabolites found in the mesenteric vein were conjugated, but only 40 % for FA. Together, the data indicated that genistein and hesperetin were more efficiently conjugated in the enterocytes than FA. However, in the liver, free FA is submitted to hepatic conjugation since the percentage of its conjugates in the extrasplanchnic blood reached 75 % of total FA (Fig. 2).

Table 1 Fate of perfused polyphenols in the splanchnic area

		Intestine			Bile
	Perfused flux	Net transfer into intestinal wall	Secretion of conjugates	Net absorption	Biliary secretion
	nmol/min	*nmol/min*			*nmol/min*
Single perfusion					
at 15 µM					
FA	12.6 ± 0.1	9.3 ± 0.6	0.6 ± 0.1	8.7 ± 0.6	0.7 ± 0.1
Gen	12.7 ± 0.6	9.8 ± 0.7	2.5 ± 0.4	7.3 ± 0.7	5.1 ± 0.2
Hesp	12.6 ± 0.5	9.7 ± 0.4	2.0 ± 0.3	7.7 ± 0.4	4.8 ± 0.5
at 120 µM					
FA	105.0 ± 5.4	84.1 ± 6.3*	3.8 ± 0.9*	80.3 ± 6.9*	5.3 ± 0.3*
Gen	94.8 ± 6.4	78.4 ± 5.0*	5.2 ± 1.0*	73.2 ± 5.1*	39.8 ± 3.2*
Hesp	107.6 ± 1.7	84.3 ± 1.7*	6.8 ± 1.1*	77.5 ± 2.7*	38.8 ± 1.9*
Combined perfusion					
FA 15 µM + Gen 15 µM					
FA	12.6 ± 0.3	9.0 ± 0.4	0.3 ± 0.1	8.6 ± 0.5	0.6 ± 0.1
Gen	12.5 ± 1.0	8.8 ± 1.1	2.3 ± 0.1	6.5 ± 1.0	4.5 ± 0.3
FA 15 µM + Hesp 15 µM					
FA	14.3 ± 0.8	9.6 ± 0.7	0.3 ± 0.1	9.3 ± 0.5	0.8 ± 0.1
Hesp	13.0 ± 0.6	10.4 ± 0.3	1.8 ± 0.3	8.6 ± 0.5	4.9 ± 0.6
Gen 15 µM + Hesp 15 µM					
Gen	12.9 ± 0.3	9.0 ± 0.6	2.3 ± 0.5	6.6 ± 0.8	4.9 ± 0.3
Hesp	10.6 ± 0.4	8.8 ± 0.4	1.5 ± 0.2	7.3 ± 0.4	4.2 ± 0.4
FA 15 µM + Gen 120 µM					
FA	12.7 ± 0.7	8.8 ± 0.9	nd	8.8 ± 0.9	0.7 ± 0.1
Gen 15 µM + FA 120 µM					
Gen	12.6 ± 0.3	10.0 ± 0.4	2.3 ± 0.4	7.7 ± 0.4	5.4 ± 0.8
Gen 15 µM + Hesp 120 µM					
Gen	12.6 ± 0.2	10.1 ± 0.2	1.2 ± 0.2*	8.9 ± 0.3*	5.0 ± 0.2

Values are means ± SEM, n = 8. The intestinal and biliary fluxes were calculated as described in the Materials and methods section. Superscripts indicate difference from the corresponding low perfused dose (15µM): *P < 0.05
FA ferulic acid; *Gen* genistein; *Hesp* hesperetin; *nd* not detectable

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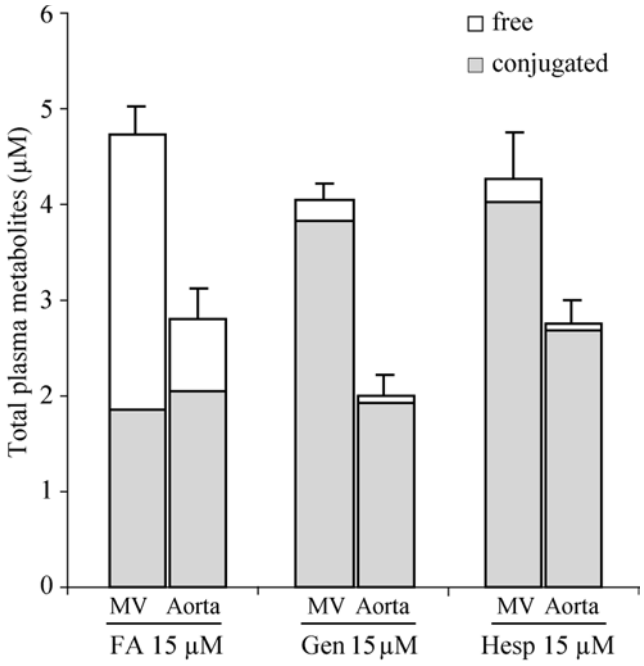

Fig. 2 Total plasma metabolites determined in the mesenteric vein and in the abdominal aorta after the perfusion of 15 μM of selected polyphenols in the small intestine. Plasma samples were analysed before and after an enzymatic treatment to hydrolyse the conjugated forms. Data are means ± SEM, n = 8

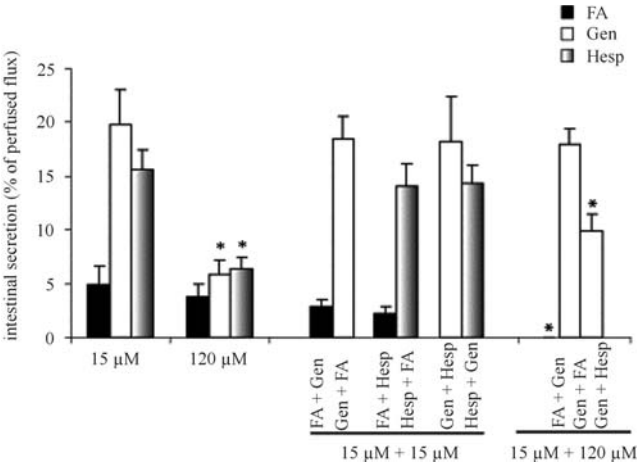

Fig. 3 Changes in the intestinal secretion of polyphenol conjugates according to the conditions of perfusion. Compounds were perfused alone (at 15 μM or at 120 μM) or in combination either at the same dose (15 μM + 15 μM) or at two different doses (15 μM + 120 μM). The intestinal secretion of conjugates was expressed in percentage of the perfused flux from the data presented in Table 1. Values are means ± SEM, n = 8. * Different from the corresponding single perfusion at 15 μM, $P < 0.05$

Influence of the perfused dose

FA, genistein and hesperetin were perfused separately in the small intestine at a concentration of 120 μM and the resulting fluxes measured in the intestinal effluent and bile were compared to those obtained previously with a dose of 15 μM (Table 1). The magnitude of the transfer of the three polyphenols through the brush border was proportional to the perfused dose. This suggests that, at least in the range of the concentrations tested, the transfer into the enterocyte did not constitute a limiting step for the intestinal absorption of polyphenols when present as aglycone.

The intestinal secretion of FA conjugates increased in proportion of the perfused dose. As shown in Fig. 3, it accounted for about 5 % of the perfused FA. Thus the net absorption of FA increased in proportion to the perfused dose (Table 1). By contrast, the rates of secretion of genistein and hesperetin conjugates into the lumen significantly declined (from 16–20 % to 6 %) when their perfused fluxes varied from 12.5 to 100 nmol/min (Fig. 3). The net absorption of genistein and hesperetin significantly increased when the perfused flux varied from 12.5 to 100 nmol/min: +20 % for genistein and +11 % for hesperetin (Table 1). For FA, genistein and hesperetin, the biliary secretion increased linearly with the perfused flux (Table 1).

Simultaneous intestinal perfusion of different polyphenols

When FA (15 μM) was perfused in combination with an equal molar amount of genistein or hesperetin, the magnitude of the net transfer in the enterocyte of each of these compounds was similar to that observed when perfused alone (Table 1). When FA was perfused together with hesperetin or genistein, the secretion of FA conjugates in the lumen tended to decline but this change was not significant (Fig. 3). Concerning genistein and hesperetin, their respective intestinal fate and biliary secretion were not affected by simultaneous perfusion at 15 μM (Table 1). These results show that, when perfused at 15 μM, the association between these polyphenols did not change their respective bioavailability.

Additional experiments have been done to assess if the presence of a high dose of a given polyphenol in the lumen may affect the intestinal uptake and metabolism of another one present at a lower dose (Table 1). When FA (15 μM) was perfused simultaneously with a high dose of genistein (120 μM), the transfer of FA in the enterocyte and of its biliary secretion was unchanged, whereas the efflux of FA conjugates in the lumen was totally suppressed. When 15 μM genistein were perfused in the presence of 120 μM hesperetin or FA, its transfer through the gut barrier and its biliary secretion were not significantly modified (Table 1). The rate of secretion of genistein conjugates in the lumen was not changed by the concomitant perfusion of FA at 120 μM. However the intestinal secretion of genistein significantly declined

(–50 %) when 15 µM genistein were perfused with 120 µM hesperetin (Fig. 3), leading to an improvement of its net absorption (+20 %) (Table 1).

In vitro conjugation of various phenolic substrates by UDP-GT

Conjugation of the three phenolic compounds by UDP-GT was compared in order to see if it could explain the differences in intestinal secretion. As shown in Fig. 4, the rate of conjugation highly varied according to the phenolic substrate. For FA, no conjugation to glucuronides could be observed. The activity of glucuronidation was 3.3-fold higher with hesperetin than with genistein (1.04 ± 0.02 vs 0.33 ± 0.02 nmol/min/mg protein respectively) (Fig. 4). When hesperetin and genistein were associated at equal dose (15 µM), the UDP-GT activity towards both molecules significantly decreased and to the same extent (–36 %). When 15 µM genistein was incubated with 120 µM hesperetin, the glucuronidation of genistein significantly decreased: –59 % compared to 15 µM genistein alone and –34 % compared to the condition where genistein and hesperetin present simultaneously at 15 µM.

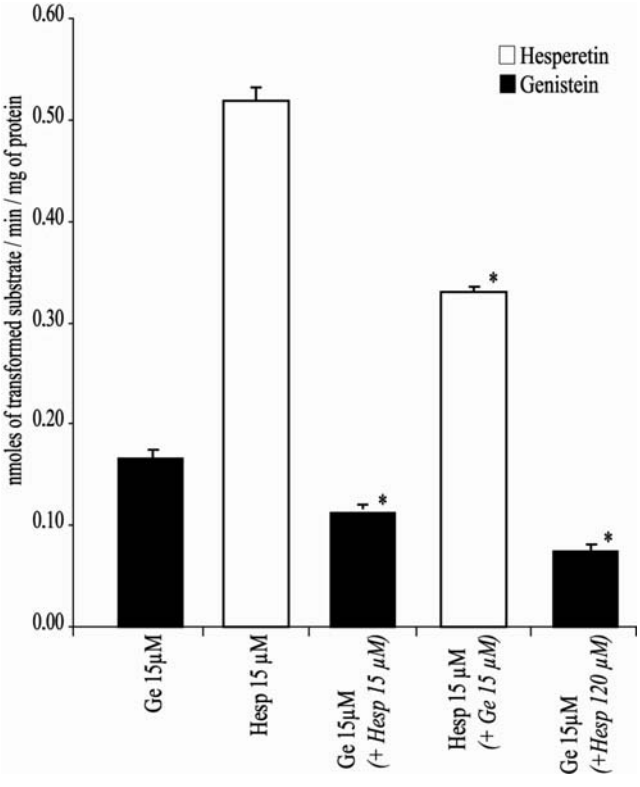

Fig. 4 *In vitro* determinations of UDP-GT activity in the presence of various phenolic substrates. FA, genistein and hesperetin, were tested alone (at 15 µM) or in combination at the same dose (15 µM + 15 µM) or at two different doses (15 µM + 120 µM). The UDP-GT activity is expressed in nmoles of transformed substrate/min/mg proteins. Values are means ± SEM. * Different from the corresponding incubation with 15 µM phenolic, $P < 0.05$; ** Different from the corresponding incubation with Gen 15 µM + Hesp 15 µM, $P < 0.05$

Respective contributions of the intestine and liver to the secretion of conjugates

From the data presented in Table 1, we have calculated the distribution of the flux of polyphenols transferred across the brush border (i. e. net transfer in the enterocyte) towards (1) intestinal secretion, (2) biliary secretion and (3) actual availability for peripheral tissues (Table 2). The obtained results are interesting because they expressed the fate in the body of the fraction of the perfused compound which gets over the brush border.

Regardless of the dose perfused or the combination tested, FA differed substantially from the two selected flavonoids by its low intestinal and biliary secretion. For genistein and hesperetin, the level of intestinal secretion of conjugates was similar, representing 20 to 25.5 % of the net transfer. This process appears saturable as shown by the significant decrease of the intestinal secretion of genistein (from 25.5 % to 6.7 % of the net transfer) in response to a shift of the net transfer from 9.8 to 78.4 nmol/min when the perfused dose varied from 15 to 120 µM. Moreover half of the decrease of the intestinal secretion of genistein conjugates observed when 15 µM genistein was perfused with a high dose of hesperetin (120 µM) accounted for competitive interactions in the enterocyte for conjugation and/or secretion of conjugates towards the lumen. In contrast, regardless of the dose or the combination between molecules, the biliary secretions of genistein and hesperetin remained at a high level, representing about 50 % of the net transfer (Table 2). Finally, due to the high rate of intestinal and biliary secretion, genistein and hesperetin are less available for peripheral tissues than FA.

Discussion

After the ingestion of a complex meal containing plant foods, various polyphenols are found as mixtures in the intestine. Possible interactions between polyphenols in the gut are explored here for the first time by in situ perfusion of rat small intestine. Among the large family of dietary polyphenols, we have selected two flavonoids, genistein and hesperetin, and one hydroxycinnamate, ferulic acid. The perfused concentrations were determined in order to reflect polyphenol intakes comparable with that provided by daily consumption of plant food in humans. Thus, the lowest concentration perfused (15 µM) could reflect an intake for humans of about 70 mg of phenolic acids or 100 mg of flavonoids per day. The highest concentration perfused (120 µM)

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Table 2 Distribution of the flux of polyphenols reaching the intestinal cells

	Net transfer into intestinal wall	Intestinal secretion	Biliary secretion	Fraction bioavailable
	nmol/min	*% of the net transfer*		
Single perfusion				
at 15 µM				
FA	9.3±0.6	6.2±1.3	7.7±0.9	86.1±4.1
Gen	9.8±0.7	25.5±3.2	51.8±3.5	22.7±1.7
Hesp	9.7±0.4	20.0±1.9	49.0±4.3	31.0±2.3
at 120 µM				
FA	84.12±6.33	4.5±1.2	6.3±1.3	89.5±3.3
Gen	78.39±5.04	6.7±1.3*	50.8±3.6	42.5±3.2*
Hesp	84.28±1.70	8.0±1.0*	46.0±1.2	46.0±2.9*
Combined perfusion				
FA 15 µM + Gen 15 µM				
FA	9.0±0.4	4.0±0.6	6.3±1.1	89.7±5.2
Gen	8.8±1.1	26.3±2.1	50.7±3.9	23.0±4.4
FA 15 µM + Hesp 15 µM				
FA	9.61±0.72	3.3±1.6	8.4±1.2	88.3±5.1
Hesp	10.40±0.30	17.3±2.1	46.8±3.3	35.9±1.7
Gen 15 µM + Hesp 15 µM				
Gen	9.0±0.6	25.9±4.2	55.2±5.2	18.9±1.5
Hesp	8.8±0.4	17.2±1.7	48.4±2.9	34.4±2.6
FA 15 µM + Gen 120 µM				
FA	8.8±0.9	nd	8.2±1.6	91.8±3.7
Gen 15 µM + FA 120 µM				
Gen	10.0±0.4	22.9±1.4	54.5±4.5	22.6±2.2
Gen 15 µM + Hesp 120 µM				
Gen	10.1±0.2	12.3±1.5*	49.4±1.3	38.3±2.1*

Values are means ± SEM, n = 8. The fractions of the net transfer secreted in the lumen and in the bile were obtained from the data presented in Table 1. Superscripts indicate difference from the corresponding low perfused dose (15 µM): *$P < 0.05$
FA ferulic acid; *Gen* genistein; *Hesp* hesperetin; *nd* not detectable

could correspond to a dose closer to the total estimated daily polyphenol intake [3].

The transfer of polyphenols through the brush border constitutes the primary step of their intestinal absorption and until now this process was unclear. The partition coefficient (log octanol/water), which reflects the lipophilicity of the molecule, has been shown to influence the permeation of polyphenols across epithelial cells [6], suggesting that passive diffusion is likely to be involved. Using the human Caco-2 cell line model, it has been proposed that the intestinal epithelial membrane transport of quercetin occurs via passive transcellular diffusion [19]. Some attempts to identify other permeation mechanisms involved in the intestinal absorption of polyphenols have been made. Using an *in vitro* mucosal uptake technique, it has been reported that a Na^+/dependent carrier-mediated uptake process was involved in the uptake of cinnamic acid across the jejunal brush border of rats [20, 21]. More recently, it has been shown that, in the presence of a proton gradient, the permeation rate of FA across Caco-2 cell monolayers was concentration dependent and saturable [22]. In the present work we first observed that for FA, genistein and hesperetin, efficiency of transfer to the enterocytes was high and proportional to the perfused dose. Secondly, the magnitude of the transfer to the enterocytes of a given compound was not affected by the simultaneous presence of another polyphenol, even if present at a higher concentration. The absence of any absorption saturation or of competitive interactions between the polyphenols does not support the existence of a specific transport system. Regardless of the system involved, our data clearly showed that the transfer to the enterocytes is not saturable in a range of concentrations commonly found in the intestine after consumption of a meal.

The presence of glucuronidated metabolites in the mesenteric blood, following the perfusion of flavonoids in the rat small intestine, shows that the enterocytes constitute the primary site for their metabolism [23–25]. For most flavonoids, a significant portion of the conjugates formed in the intestinal cells is secreted back to the lumen, thus reducing the net absorption [6]. Previous

studies strongly suggested that the active secretion of polyphenol conjugates in the lumen may be governed by MRP-2 and/or the P-glycoprotein [26, 27]. In the present work, the rate of secretion of conjugates back into the lumen differed markedly according to polyphenols. Secretion was low for FA, compared to the two flavonoids tested. This difference may be linked to the low intestinal metabolism of FA, as suggested by the inability of UDP-GT to catalyse FA glucuronidation and by the high level of free FA in the mesenteric vein. In contrast to FA, genistein and hesperetin were good substrates of UDP-GT resulting in a high secretion of conjugated metabolites in the intestinal lumen as described previously [28, 29].

The level of the intestinal secretion of conjugates was not changed by the concomitant perfusion of low doses of different polyphenols (15 μM) in the enterocytes, whereas it markedly decreased when the perfused dose of flavonoids increased. This may reflect an efficient intestinal conjugation at low concentrations and a saturation of the intestinal conjugation at high concentrations. Moreover, some competition between flavonoids for their conjugation may occur when competing substrates are present at high dose. This possibility is suggested by the changes in the UDP-GT activities obtained after an incubation with a mixture of genistein and hesperetin compared to single substrate. However, the possibility of a competition between flavonoids for the transport systems involved in the efflux of their conjugates in the lumen cannot be ruled out.

The fraction of intestinal conjugates escaping re-excretion into the lumen corresponds to the net absorption, but not to the fraction finally available for peripheral tissues because hepatic uptake and metabolism is very active and conjugates may be secreted into the bile [30–33]. As shown in the present work, the intensity of biliary secretion may substantially differ among polyphenols. Because genistein and hesperetin were highly secreted in the bile, their final bioavailability for peripheral tissues was markedly lower than that of FA. However such a difference in final bioavailabilities was not obvious when the aortic plasma concentrations in each polyphenol were taken into account (Fig. 2). Even if we lack some experimental data to explain this discrepancy, some hypothesis may be considered. First, this may result from a rapid and efficient urinary excretion of FA after its delivery in aortic plasma. Besides, following an oral single dose administration of FA to rats, the peak plasma concentration of FA was found at 30 min and within 1.5 h 43 % of the total metabolites of FA were excreted in urine [34]. By contrast, the urinary excretion of both genistein and flavanones appeared lower and slow [35, 36], in agreement with the importance of their enterohepatic cycling observed in our study. Taken together these data may explain why the aortic plasma levels of the three polyphenols are not significantly different after a 45 min perfusion period. On the other hand, FA is a small molecule compared to genistein or hesperetin; thus FA conjugates could more easily enter tissues than those of genistein or hesperetin.

The extent of enterohepatic cycling of the tested polyphenols was proportional to the perfused dose and unaffected by the simultaneous presence of different compounds in the intestine. Thus, according to the nature of the compound, the biliary secretion of conjugates may largely determine the availability of polyphenols for peripheral tissues. Even if the active biliary excretion of flavonoids reduces their net absorption, the enterohepatic cycling could maintain high circulating levels by spreading absorption over a longer period.

In conclusion, the intestinal absorption and the splanchnic metabolism of polyphenols largely govern their bioavailability. Intestinal and biliary secretion of genistein and hesperetin conjugates was high and that of FA relatively low, resulting in a high bioavailability of FA.

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