Research Article

The Ussing type chamber model to study the intestinal transport and modulation of specific tight-junction genes using a colonic cell line

Hannah Bergmann[1]*, Dorothee Rogoll[2]*, Wolfgang Scheppach[2, 3], Ralph Melcher[2], Elke Richling[1]

[1] Department of Chemistry, Division of Food Chemistry and Toxicology, University of Kaiserslautern, Kaiserslautern, Germany
[2] Department of Medicine II, Division of Gastroenterology, University of Wuerzburg, Wuerzburg, Germany
[3] Juliusspital Wuerzburg, Wuerzburg, Germany

Polyphenols in apples, such as various hydroxycinnamic acids and flavonoids, have positive health effects that strongly depend on their bioavailability. In order to show that the Ussing-type chamber is a useful model to study metabolism, transport, and tightness of cell monolayers in one experimental setup, monolayers of the T84 colon carcinoma cell line mounted in Ussing-type chambers were incubated in the presence of physiological concentrations of various hydroxycinnamic acids (including ferulic, isoferulic, cinnamic, and hydrocinnamic acids) and flavonoids for 4 h. Concentrations of each tested polyphenol in the apical chamber, basolateral chamber, and those associated with the cells were then determined using HPLC with DAD (HPLC-DAD). The transport studies showed that the amounts of the tested polyphenols that passed from the apical to the basolateral side of the T84 monolayers depended on their polarity. Metabolites, such as glucuronides and sulfates of ferulic acid, were also detected at measurable levels by HPLC-ESI-MS/MS in the model system, but only when they were supplied at supra-physiological concentrations (>100 μM). In addition, the transepithelial resistance (TER) of T84 monolayers was measured before and after the addition of polyphenols, with and without short-term exposure to apical sodium caprate (C10), a tight junction (TJ) modulator. Exposure to C10 induced a decrease in TER that was reversible by incubation with polyphenols. However, no increase in paracellular permeability of tested polyphenols was observed after apical C10 exposure, so C10 did not promote fluxes of hydroxycinnamic acids across the monolayers. Further, real-time PCR analysis of the T84 colon cell line showed that ferulic and isoferulic acids induced significant increases in expression of the TJ components zonula occludens-1 (ZO-1) and claudin-4 transcription, but reductions in occludin expression. In contrast, caffeic and *p*-coumaric acids had no significant effects on the transcription of either ZO-1 or occludin. Our results provide confirmation that T84 cells could be used as model system to simulate the intestinal mucosa, and that polyphenols are able to increase the TER of C10-treated and -untreated T84 monolayers.

Keywords: Apple juice / Hydroxycinnamic acids / Polyphenols / T84 / Tight junctions

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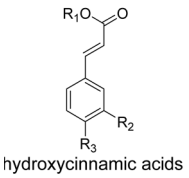
1 Introduction

Hydroxycinnamic acids and their derivatives (Fig. 1) are polyphenols that play important roles in secondary plant metabolism and therefore, are widely distributed in both plants and diverse plant-based foods (*inter alia*) grains, fruits, coffee, and red wine [1]. In apples and apple products, for instance, hydroxycinnamic acids are found together with the glycosides of the flavonol quercetin and

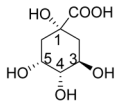
Correspondence: Dr. Elke Richling, Department of Chemistry, Division of Food Chemistry and Toxicology, University of Kaiserslautern, Erwin-Schroedinger-Str. 52, 67663 Kaiserslautern, Germany
E-mail: richling@chemie.uni-kl.de
Fax: +49-631-205-4398

Abbreviations: C10, sodium caprate; **GAPDH,** glyceraldehyde-3-phosphate dehydrogenase; **HBSS,** Hanks' balanced salt solution; **MCT,** monocarboxylic acid transporter; **MDR1,** P-glycoprotein; **MRP,** multidrug resistance protein; **TER,** transepithelial resistance; **TJ,** tight junction; **ZO-1,** zonula occludens-1

* Both authors contributed equally to this work.



hydroxycinnamic acids



D-(-)-quinic acid

	R_1	R_2	R_3	incubation concentration (μM)
cinnamic acid **1**	H	H	H	20
p-coumaric acid **2**	H	H	OH	20
caffeic acid **3**	H	OH	OH	20
ferulic acid **4**	H	OCH_3	OH	20
isoferulic acid **5**	H	OH	OCH_3	20
4-*p*-coumaroylquinic acid **6**	quinic acid (4)	H	OH	20
1-caffeoylquinic acid **7**	quinic acid (1)	OH	OH	10
5-caffeoylquinic acid **8**	quinic acid (5)	OH	OH	50
methyl *p*-coumarate **9**	CH_3	H	OH	20
methyl caffeate **10**	CH_3	OH	OH	20

Figure 1. Chemical structures and concentrations of hydroxycinnamic acids used in the experiments with the Ussing chambers and T84 colon cell model. Numbers in brackets indicate the esterification positions of D-(−)-quinic acid.

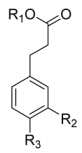
the dihydrochalcone phloretin. Further, hydroxycinnamic acids and flavonoids reportedly have various desirable health attributes, including antioxidative and anticarcinogenic properties, and positive effects on diabetes, neurodegenerative, and cardiovascular diseases [2].

To assess such biological effects, it is important to acquire knowledge of the bioavailability and metabolism of these compounds following ingestion. The availability of polyphenols in the gastrointestinal tract of humans has been evaluated in studies in which cloudy apple juice was consumed by ileostomists, and up to 30% of the oral dose was recovered in their ileostomy bags [3]. Metabolites of the ingested polyphenols, such as D-(−)-quinic acid, the methyl esters of caffeic acid and *p*-coumaric acid, 1-caffeoylquinic acid, 3-caffeoylquinic acid, phloretin 2′-*O*-glucuronide, and the aglycone phloretin were also recovered in the effluents [4]. These studies have provided indications of colonic concentrations of polyphenols following the consumption of apples, which can be used in physiologically relevant investigations of their transcellular fluxes and metabolism in the intestinal epithelium.

Several attempts have been made to develop models based on human intestinal cell lines that mimic the functional and morphological organization of the intestinal epithelium. The most commonly used models for *in vitro* studies of intestinal absorption and metabolism of xenobiotics and flavonoids have been systems constructed using the Caco-2 cell line [5]. Notably, transport of the hydroxycinnamic acid ferulic acid across a cell monolayer of cocultured Caco-2 and HT29-MTX cells, and the formation of several conjugates (such as feruloyl−sulfate, feruloyl−glucuronide, and hydroferulic acid), have been recently observed by Poquet *et al*. [6]. Studies by other groups have also shown that *p*-coumaric, 5-caffeoylquinic, and caffeic acids can be transported across a Caco-2 monolayer, and the presence of a proton gradient enhances their transport [7, 8]. Further, methyl hydroxycinnamates may be converted into the corresponding free acids and their glucuronides or sulfates by cellular metabolism [9], and the flavonol quercetin is transported across the Caco-2 monolayer both from the apical to the basolateral side and *vice versa* [10]. In contrast, no transport of green tea catechins, such as (−)-epicatechin, across Caco-2 monolayers from apical to basolateral compartments was detected, although their transport from the basolateral to the apical side was observed, together with metabolites of (−)-epicatechin such as glucuronides and sulfates, in studies by Zhang *et al*. [11]. Several ATP binding cassette (ABC) transporter proteins such as P-glycoprotein (MDR1), multidrug resistance protein 2 (MRP2), breast cancer resistance protein (BCRP), monocarboxylic acid transporter 1 (MCT1), and sodium-dependent monocarboxylic acid transporter (S-MCT) are present in the Caco-2 cell line and are involved in transport of polyphenols [6, 12]. Although the human colonic carcinoma cell line T84, has been widely employed in functional and molecular analyses of intestinal epithelia [5] knowledge about the presence of transport proteins is very limited. Expression of mRNA of MDR1 and MRP2 was observed by Haslam *et al*. [13]. Protein expression could be detected by Western blot analysis for MDR1 whereas MRP2 was not detectable [14].

The transfer of molecules such as polyphenols and flavonoids between cells of epithelia (paracellular transport) in vertebrates is regulated to a large degree by tight junctions (TJs), composed of a branching network of sealing strands. These junctions maintain apical and basolateral polarization by providing a barrier that separates the interior and exterior compartments and restricts the lateral diffusion of pathogens, cell membrane proteins, and soluble substances, with varying degrees of charge and size selectivity. Thus, paracellular permeability is heavily dependent on the number of strands and hence the expression of key protein components of TJs, notably claudins [15]. The permeability of TJs is also physiologically modulated by diverse agents including (*inter alia*) phorbol esters, proinflammatory cytokines, Ca^{2+}, ATP, capric acid, and bacterial toxins [16–20]. Intestinal inflammation in patients with inflammatory bowel disease, on the other hand, results in dysregulation of TJs [21–23]. Thus, knowledge of the regulation of TJs, and whether or not natural food compounds may counteract pathological influences, is of major importance.

We hypothesized that hydroxycinnamic acids and flavonoids may affect epithelial barrier functions, and postulated that this hypothesis could be tested using the human colonic



	R_1	R_2	R_3	incubation concentration (μM)
hydrocinnamic acid **11**	H	H	H	20
hydrocaffeic acid **12**	H	OH	OH	20
hydroferulic acid **13**	H	OCH_3	OH	20

hydrohydroxycinnamic acids

Figure 2. Chemical structures and concentrations of hydrohydroxycinnamic acids used in the experiments with the Ussing chambers and T84 cells.

carcinoma cell line T84 [5]. T84 cells show structural similarities to intestinal crypt cells and their TJ strand numbers show increases with time in monolayer cultures that correlate with increases in their resistance [24].

Since intestinal crypt cells are believed to be leakier than surface cells it is unclear how the T84 monolayer culture model relates to the differentiation of cells *in vivo*, but it may be representative of the more differentiated epithelium in terms of TJ assembly. In the study presented here we investigated the transport, metabolism, and influence on the expression of TJ-related genes of various hydroxycinnamates and flavonoids (for structures, see Figs. 1 and 2) in T84 model cell cultures in Ussing chambers (which are well established for *in vitro* studies of intestinal ion transport, drug absorption, and permeability to variously sized marker molecules and antigens in gut mucosa [25]). Polyphenol concentrations used in the incubations were comparable to physiological concentrations found in ileostomy bags of patients following consumption of cloudy apple juice [3]. Several groups have reported that some polyphenols are unstable under cell culture conditions [26–28]. Therefore, we also assessed the stability of the polyphenols in the incubation buffer used in the transport experiments. In addition, we assessed the effects of the polyphenols on the transepithelial resistance (TER) of the monolayers, both with and without short-term exposure to apical sodium caprate (C10), a TJ modulator [29].

2 Materials and methods

2.1 Chemicals

All chemicals and solvents were of analytical grade. ACN (gradient grade) was purchased from Baker (Deventer, The Netherlands). Ferulic acid 4 (4-hydroxy-3-methoxycinnamic acid), isoferulic acid 5 (3-hydroxy-4-methoxycinnamic acid), D-(−)-quinic acid, and the internal standard 3,4,5-trimethoxycinnamic acid were purchased from Aldrich (Steinheim, Germany). Caffeic acid 3 (3,4-dihydroxycinnamic acid), 5-caffeoylquinic acid 8, phloretin 14 (2′,4′,6′,4-tetrahydroxydihydrochalcone), phloretin 2′-*O*-glucoside 15, (−)-epicatechin 19 (*cis*-5,7,3′,4′-tetrahydroxyflavan-3-ol; 2R, 3R), and 3,4-dihydroxybenzoic acid 17a were from Sigma (Steinheim, Germany). Quercetin 17 (3,5,7,3′,4′-pentahydroxyflavone) was from Merck (Darmstadt, Germany). *p*-Coumaric acid 2 (4-hydroxycinnamic acid), cinnamic acid 1, and hydrocaffeic acid 12 were purchased from Fluka (Steinheim, Germany). (+)-Catechin 20 (*trans*-5,7,3′,4′-tetrahydroxyflavan-3-ol, 2R, 3S) and quercetin 3-*O*-rhamnoside 8 were purchased from Roth (Karlsruhe, Germany). Hydrocinnamic acid 11 was purchased from Acros Organics (Geel, Belgium). Hydroferulic acid 13 was provided by ABCR (Karlsruhe, Germany). 1-Caffeoylquinic acid 7 was synthesized from caffeic acid and D-(−)-quinic acid according to the published protocols [30, 31]. 4-*p*-Coumaroylquinic acid 6 and phloretin 2′-*O*-xyloglucoside 16 were isolated from extracts of apple juice treated with laccase [32]. Methyl caffeate 10 and methyl *p*-coumarate 9 were kindly provided by P. Schreier (Wuerzburg, Germany).

2.2 Stability studies of polyphenols

Polyphenols (10–50 μM) were incubated in Hanks' balanced salt solution (HBSS) (pH 7.4) in Falcon tubes (Greiner Bioone, Frickenhausen, Germany) without cells for 24 h at 37°C. After 0, 2, 4, 6, 8, and 24 h portions of the mixtures (300 μL) were analyzed by HPLC with DAD (HPLC-DAD) and HPLC-ESI-MS/MS as described in Sections 2.3 and 2.4.

Quercetin was incubated at 37°C for 1 h with HBSS pregassed with $^{18}O_2$ (Sigma–Aldrich, Steinheim, Germany). The solution was lyophilized and the residue was measured by HPLC-ESI-MS/MS in both full scan mode and product ion mode (*m*/*z* 319; 30 eV) with the MS parameters listed in Section 2.4.

2.3 HPLC-DAD analysis

HPLC-DAD analysis was performed using an Agilent 1200 HPLC system, with the column and autosampler heaters, respectively, set at 30 and 4°C, equipped with a Zorbax C18 column (Agilent, Waldbronn, Germany). Ten microliters of each sample was injected and separated by a mobile phase consisting of a 40 min linear gradient of 1–50% ACN, balanced by aqueous 0.1% formic acid, with a flow rate of 1 mL/min. Eluting dihydrochalcones and catechins were detected at 280 nm, hydroxycinnamic acid derivates at

320 nm and flavonols at 360 nm. LOQs and LODs (defined as three and five times the S/N, respectively) ranged from 0.04 to 1.0 mg/L, and from 0.02 to 0.4 mg/L, respectively.

2.4 HPLC-MS/MS analysis

For HPLC-MS/MS analysis, an SCIEX API 3200 MS/MS tandem mass spectrometer equipped with an electrospray (ESI) interface (Applied Biosystems, Darmstadt, Germany) coupled to a Jasco HPLC system with two pumps (PU-2080) and a thermostated autosampler (AS-2057) (Jasco, Groß-Umstadt, Germany) was used. Data were acquired and evaluated using Analyst Software 1.4.2 (Applied Biosystems). The chromatographic conditions were the same as those described above. ESI settings were as follows: negative ionization mode; spray capillary voltage, 4.5 kV; curtain gas, nitrogen (450°C at 25 psi); GS1, 50 psi; GS2, 40 psi; declustering potential, –40 V; entrance potential, –4.5 V; electron multiplier voltage, 2.2 kV. The mass spectrometer was operated in full scan mode (120–900 U), with a total scan duration of 1.0 s and dwell time of 2 ms. MS/MS experiments were performed at a collision energy of 20 eV, with nitrogen (6 U) serving as collision gas. The molecular ion peaks and product ion spectra obtained were compared to those of authentic references.

2.5 Cell culture and TER analysis

T84 colon epithelial cells purchased from American Type Culture Collection (ATCC, Rockville, MD, USA; CCL-248) were grown in a 5% CO_2 humidified incubator at 37°C on 1 μm filters (Falcon, Heidelberg, Germany) with medium containing Ham's F-12 nutrient mixture and Dulbecco's minimum essential medium (DMEM) (1:1) supplemented with 5% fetal calf serum, antibiotics (PenStrep), and 2.5 mM glutamine (all from Gibco, Germany). The TER of the resulting monolayers was determined in an Endohm 24 chamber (WPI, Berlin, Germany). In all experiments, we utilized T84 monolayers of at least 2 wk confluence when the TER was reproducible, and the monolayer was intact. Cytotoxicities of the polyphenols were tested in appropriate concentrations by trypan blue staining [33].

2.6 Taqman® probes

Taqman probes were synthesized together with oligonucleotides by MWG (Ebersberg, Germany). VIC-labeled probes for the specific Taqman housekeeping control gene, human glyceraldehyde-3-phosphate dehydrogenase (GAPDH), were purchased from Applied Biosystems. The following primers and probes were designed using Beacon Designer 2.1 (Premier Biosoft, Palo Alto, USA) and overt amplification of DNA: zonula occludens-1 (ZO-1) sense, 5′-ATG GTG TCC TAC CTA ATT CAA CTC AT-3′; ZO-1 antisense, 5′-GCC AGC TAC AAA TAT TCC AAC ATC A-3′; ZO-1 probe, 5′-CAC CAG CCA GCC GCA AAC CCA CA-3′; occludin sense, 5′-AAG GTC AAA GAG AAC AGA GCA AGA-3′; occludin antisense, 5′-TAT TCC CTG ATC CAG TCC TCC TC-3′; and OCC probe, 5′-CTC ATC ACA GGA CTC GCC GCC AGT TG-3′. Probes were labeled with 5′-FAM and 3′-TAMRA. Claudin-4 oligonucleotides and probes were measured with a "ready-to-use" kit purchased from Applied Biosystems.

2.7 Real-time PCR

Total RNA from T84 monolayers grown on culture inserts was obtained by lysis using TriFast (Peqlab, Erlangen, Germany) and purified using RNeasy columns (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Portions (2 μg) of RNA were reversely transcribed using iScript (BioRad, Munich, Germany) then amplified by real-time PCR using an iCycler (BioRad). After activating the polymerase at 95°C for 10 min the ZO-1, occludin, claudin-4, and GAPDH primers and probes were annealed at 60°C, and transcript levels were semiquantitatively analyzed, using standard calibration curves obtained from serial dilutions of cDNAs synthesized from known quantities of total RNA from T84 cells. ZO-1, occludin, claudin-4, and GAPDH starting transcript number values for the standard curves were set arbitrarily. ZO-1, occludin, claudin-4, and GAPDH expression levels were then estimated in terms of cycle threshold (C_t) values, and corresponding transcript numbers were read off standard curves as previously described [34]. Values in unknown samples were quantified by measuring C_t and reading the corresponding values of the standard curves. ZO-1, occludin, and claudin-4 expression levels were then normalized to GAPDH expression and their expression levels in polyphenol-free medium-treated control cells were considered to be "1." All experiments were conducted in triplicates.

2.8 Ussing chamber experiments

For Ussing chamber experiments with the T84 colon carcinoma cell line, cells were grown in 12 mm diameter permeable transwell cell culture inserts (Corning, Wiesbaden, Germany). After cultivating the cells until confluency these permeable supports were fixed in P2302 Ussing chamber inserts (Hugo Sachs Elektronik, Harvard Apparatus, March-Hugstetten, Germany), exposing 0.186 cm^2 of cell surface to 5 mL of HBSS (Sigma, Munich, Germany) and kept at 37°C under constant 95% O_2–5% CO_2 gassing. After a 30-min equilibration period to achieve steady-state conditions each of the test polyphenols was added, individually, to apical compartments of the chambers, at concentrations listed in Table 1 and Figs. 1 and 2 in DMSO (final DMSO concentration, 0.05%). The transepithelial electrical potential difference was then measured at 2 min intervals under current-clamped conditions. Tissue conductance or TER was deter-

Table 1. Flavonoids and their concentrations (μM) used in transepithelial transport experiments in the Ussing chambers with colon cell line T84

Compound	Concentrations (μM)
Phloretin 14	20
Phloretin 2′-*O*-glucoside 15	20
Phloretin 2′-*O*-xyloglucoside 16	20
Quercetin 17	10
Quercetin 3-*O*-rhamnoside 18	10
(−)-Epicatechin 19	10
(+)-Catechin 20	10

mined at an applied current of 100 mA, and the short-circuit current (Isc) was calculated using Ohm's law ($R = V/I$). Samples of 0.5 mL were taken every 60 min from both sides of the chamber, frozen in liquid nitrogen, and stored at −80°C until measurement. Cell suspensions were obtained by lysis using TriFast reagent (Peqlab). All experiments were done in triplicate. In order to detect changes in permeability and the influence of the polyphenols on the colonic barrier, the TJ modulator C10 (Sigma) was added to the apical side for approximately 10 min, following which the TER was *ca*. 50% of its initial value. The concentration of C10 applied (10 mM) has been reported to increase the paracellular permeability of both human and rat ileum [20]. In these experiments, the C10 was washed away using fresh HBSS medium after the 10 min incubation period before adding the test polyphenols.

In additional experiments, ferulic acid was incubated with T84 cells at concentrations of 20, 50, 100, 500, and 1000 μM, according to Poquet *et al*. [6].

2.9 Sample preparation prior to HPLC-DAD analysis

For HPLC-DAD analysis, apical and basolateral samples were thawed rapidly at 20°C and the internal standard 3,4,5-trimethoxycinnamic acid was added. Cell suspensions were thawed in a sonication bath for 20 min, then 200 μL portions of the suspensions were passed by centrifugation through a 3 kDa membrane (Nanosep 3K omega from Pall, Michigan, USA), at 10 000 rpm and 4°C for 5 min to dryness. The membrane was then washed once with 100 μL and twice with 50 μL formic acid in methanol (10:90 v/v) and centrifuged as described above. Then 3,4,5-trimethoxycinnamic acid was added to the combined eluates and the samples were analyzed using HPLC-DAD (as described in Section 2.3). Peak identities were confirmed by HPLC-ESI-MS/MS (as described in Section 2.4).

2.10 Statistics

Statistical evaluations were performed using Student's paired *t*-tests, and differences were considered significant if $p \leq 0.05$. Data are presented as means ± standard deviation (SD).

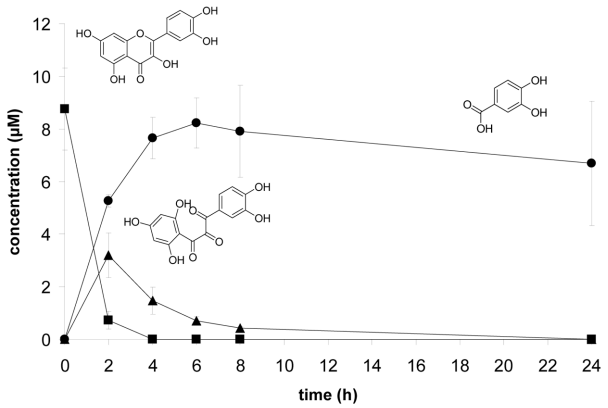


Figure 3. Degradation of quercetin in HBSS buffer at 37°C and formation of 3,4-dihydroxybenzoic acid and 2,5,7,3′,4′-pentahydroxy 3,4-flavandione. ■, Quercetin; ●, 3,4-dihydroxybenzoic acid; ▲, 2,5,7,3′,4′-pentahydroxy 3,4-flavandione. Each point is the mean ± SD obtained from three experiments.

3 Results

3.1 Stability of polyphenols in HBSS

The stability of some of the incubated polyphenols (at concentrations shown in Table 1 and Figs. 1 and 2) in HBSS buffer at 37°C, in the absence of cells, was investigated, and the results are shown in Table 2. *p*-Coumaric acid 2, ferulic acid 4, methyl *p*-coumarate 9, hydroferulic acid 13, phloretin 2′-*O*-glucoside 15, and quercetin 3-*O*-rhamnoside 18 were stable over 4 h. Compounds 2, 9, 13, and 15 remained stable in HBSS buffer over 24 h, whereas 4 and 18 were partially degraded. Caffeic acid 3, 5-caffeoylquinic acid 8, methyl caffeate 10, hydrocaffeic acid 12, phloretin 14, (−)-epicatechin 19, and (+)-catechin 20 were partially degraded within 4 h. Quercetin 17 was not detectable after 4 h of cell-free incubation. Further, two degradation products of quercetin 17 were detected by HPLC-DAD and HPLC-ESI-MS/MS after incubation in HBSS buffer at 37°C (Fig. 3). The identity of 3,4-dihydroxybenzoic acid 17a was confirmed by comparing its retention time, UV-spectrum (λ_{max} 260 nm) and HPLC-ESI-MS/MS spectrum with that of the reference compound. The degradation product 17b was more polar than the incubated aglycone 17 (retention times, 15.4 and 26.6 min, respectively) and showed a shifted UV-spectra with an absorption maximum at 295 nm. HPLC-ESI-MS/MS-spectra of its molecular ion (*m*/*z* 317) indicated that the metabolite 17b was 2,5,7,3′,4′-pentahydroxy 3,4-flavandione. To confirm this finding 17 was incubated with $^{18}O_2$ saturated HBSS, resulting in the formation of 17b with – in this case – two molecular peaks (*m*/*z* 317 and 319). Their product ion spectra revealed an

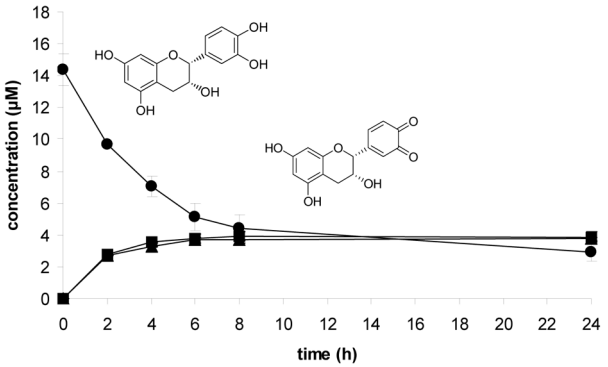


Figure 4. Degradation of (−)-epicatechin in HBSS buffer at 37°C and formation of two (−)-epicatechin quinones. ●, (−)-Epicatechin; ■, quinone a; ▲, quinone b. Each point is the mean ± SD obtained from three experiments.

identical fragment of *m*/*z* 151, corresponding to the A-ring, whereas the fragments corresponding to the B-ring differed by 2 amu (*m*/*z* 227 and 255 for 17b and *m*/*z* 229 and 257 for ^{18}O-17b) (data not shown) [35]. The two degradation products appeared after 2 h, and the concentrations of 17a and 17b peaked after 2 h and between 4 and 8 h, respectively.

The flavan-3-ol (−)-epicatechin 19 was degraded in the HBSS incubation buffer, leading to the simultaneous formation of two products (19a and 19b) with identical MS/MS spectra and UV maxima (Fig. 4). Further, while 19 showed a single UV absorption peak at 280 nm, 19a and 19b each displayed an additional peak at 253 nm. Their additional UV peaks and MS/MS spectra (including a deprotonated ion at *m*/*z* 287) indicate that 19a and 19b represent two diastereomeric (−)-epicatechin quinones.

3.2 T84 transport studies in the Ussing chamber

To assess the transport of polyphenols across T84 monolayers, the test polyphenols were added, individually, into the apical compartments of Ussing-type chambers containing monolayers after 30 min equilibration with the assay buffer. Concentrations and metabolites of the tested compounds in the apical and basolateral chambers, and the cell suspension, were then analyzed by HPLC-DAD and HPLC-ESI-MS/MS at five timepoints during the following 4 h. The polyphenols did not show any cytotoxicity toward the T84 cells in the tested concentrations according to trypan blue staining assays.

3.3 Transport of hydroxycinnamic acids and their esters

Assays with the hydroxycinnamic acids and their derivatives, at concentrations ranging between 10 and 50 μM (in accordance with reported intestinal concentrations following apple juice consumption, see Fig. 1), indicated that 29.0, 24.8, 2.3, and 3.2% of the free acids cinnamic acid 1,

Table 2. Stability of polyphenols in HBSS at 37°C

Compound	% of initial concentration	
	4 h	24 h
p-Coumaric acid 2	103.3 ± 2.7	107.1 ± 3.5
Caffeic acid 3	29.3 ± 6.0	3.0 ± 0.5
Ferulic acid 4	99.1 ± 0.9	88.0 ± 2.1
5-Caffeoylquinic acid 8	61.2 ± 6.3	20.6 ± 1.3
Methyl *p*-coumarate 9	101.0 ± 1.7	98.9 ± 1.8
Methyl caffeate 10	76.9 ± 1.1	37.4 ± 1.8
Hydrocaffeic acid 12	33.5 ± 3.4	4.6 ± 0.7
Hydroferulic acid 13	112.5 ± 4.9	110.7 ± 0.1
Phloretin 14	85.7 ± 4.2	44.0 ± 8.4
Phloretin 2′-*O*-glucoside 15	100.9 ± 0.7	96.5 ± 0.4
Quercetin 17	n.d.	n.d.
Quercetin 3-*O*-rhamnoside 18	93.3 ± 2.6	55.6 ± 5.5
(−)-Epicatechin 19	49.1 ± 0.8	20.1 ± 1.7
(+)-Catechin 20	89.2 ± 4.5	47.3 ± 0.3

Percentages of the initial concentration remaining after 4 and 24 h. Each value is the mean ± SD obtained from two experiments, for details see Section 2.
n.d., not detected.

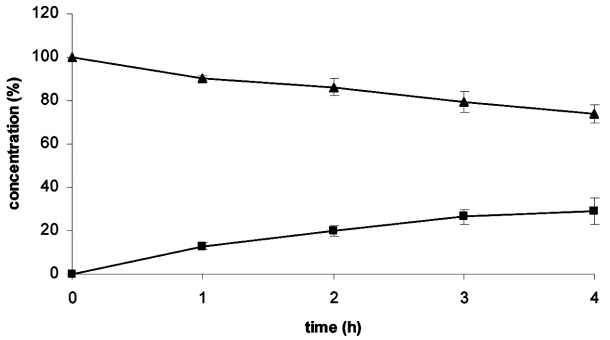


Figure 5. Time courses of percentage changes in the concentration of cinnamic acid in the apical compartment (▲, initial concentration 20 μM) and basolateral compartment (■), in incubations with T84 cell monolayers illustrating its transepithelial transport. Each point indicates the mean ± SD obtained from three experiments.

hydrocinnamic acid 11, ferulic acid 4, and isoferulic acid 5, respectively, were transported from the apical to the basolateral side of the T84 monolayers during 4 h incubation (Table 3). In addition, 45.8 and 37.2% of the initial amounts of the methyl esters of *p*-coumaric 9 and caffeic acid 10 were transported through the monolayers. In contrast, no transport to the basolateral side was detected for the more hydrophilic *p*-coumaric 2, caffeic 3, hydrocaffeic 12, hydroferulic 13 acids, or the esters 4-*p*-coumaroylquinic acid 6, 1-caffeoylquinic acid 7, and 5-caffeoylquinic acid 8. Amounts (ranging between 0.2 and 1.1% of the initial concentrations) of 1, 4, 5, 8, 9, and 10 were detected by HPLC-ESI-MS/MS in the cell suspensions (together with sub-LOQ levels of 2, 3, 7, 11, 12, and 13) and quantified by HPLC-DAD. No metabolites of the hydroxycinnamic acids were detected in the cell suspensions or either the apical or

Table 3. Transepithelial transport experiments

Compound	Percentages (%) recovered		
	Apical	Basolateral	Cell associated
Cinnamic acid 1	74.1 ± 3.4	29.0 ± 4.9	1.1 ± 0.1
p-Coumaric acid 2	109.7 ± 1.7	n.d.	Traces
Caffeic acid 3	82.9 ± 6.6	n.d.	Traces
Ferulic acid 4	102.7 ± 4.4	2.3 ± 0.2	0.9 ± 0.1
Isoferulic acid 5	102.4 ± 12.8	3.2 ± 2.5	0.3 ± 0.1
4-*p*-Coumaroylquinic acid 6	90.2 ± 4.4	n.d.	n.d.
1-Caffeoylquinic acid 7	94.9 ± 3.6	n.d.	Traces
5-Caffeoylquinic acid 8	104.9 ± 9.1	n.d.	0.2 ± 0.1
Methyl *p*-coumarate 9	55.6 ± 0.5	37.2 ± 2.3	0.9 ± 0.1
Methyl caffeate 10	62.8 ± 0.7	45.8 ± 3.1	1.1 ± 0.1
Hydrocinnamic acid 11	67.2 ± 5.8	24.8 ± 0.6	Traces
Hydrocaffeic acid 12	23.2 ± 6.3	n.d.	Traces
Hydroferulic acid 13	115.0 ± 10.1	n.d.	Traces

Distribution of hydroxycinnamic acids in the cell suspensions and both the apical and basolateral sides of the Ussing chambers and T84 monolayer transepithelial transport model after 4 h incubation. Each value is the mean ± SD obtained from three experiments, as detailed in Section 2.
n.d., not detected.

Table 4. Transepithelial transport experiments

Concentrations (μM)	Percentages (%) recovered		
	Apical	Basolateral	Cell associated
20	102.7 ± 4.4	2.3 ± 0.2	0.9 ± 0.1
50	86.8 ± 3.8	2.1 ± 0.1	0.8 ± 0.1
100	96.6 ± 2.1	2.1 ± 0.1	0.9 ± 0.1
500	100.9 ± 5.5	1.8 ± 0.8	0.5 ± 0.1
1000	94.7 ± 6.3	2.7 ± 0.8	0.3 ± 0.1

Distribution of ferulic acid 4 in the cell suspensions and both the apical and basolateral sides of the Ussing chambers and T84 monolayer transepithelial transport model after 4 h incubation. Each value is the mean ± SD obtained from three experiments, as detailed in Section 2.

basolateral compartments. To illustrate the transport kinetics of these substances, time courses of levels of cinnamic acid in the two Ussing chamber compartments are shown in Fig. 5.

3.4 Transport and metabolism of ferulic acid

As shown in Table 4, ferulic acid 4 was detected in the cell suspensions and both the apical and basolateral compartments when it was incubated in the model system at physiological (20 μM) and higher concentrations (50 – 1000 μM). The proportions transported to the basolateral compartment were similar at all test concentrations. Following incubation of the cells with 1000 μM of 4 metabolites (five in total) were also detected in the cell suspensions and in both compartments by HPLC-DAD and HPLC-ESI-MS/MS. The identities of three of these metabolites – feruloyl glucuronide 4a, feruloyl sulfate 4b, and hydroferulic acid 13 (*m*/*z* ratios of molecular ions: 369, 273, and 195, respectively) – were confirmed by comparison of their relative retention times and product ions with those of authentic standards (Table 5), according to Poquet *et al*. [6]. For 4a (retention time, 12.3 min), the main diagnostic ferulic acid fragment, with an *m*/*z* ratio of 192.9, was formed by the neutral loss of glucuronic acid (176 U, data not shown). For 4b, the *m*/*z* 192.9 product ion was obtained at a retention time of 14.0 min, and a product ion with *m*/*z* 135.9 was confirmed to be 13. The fragmentation patterns obtained were consistent with previously reported patterns [6]. The fragmentation pattern and retention time of hydroferulic acid were also compared to those of a commercially available standard. Of the other three metabolites (4c, 4d, and 4e) detected after incubation with 4, 4c (*m*/*z* 307) was only detectable in the cell suspension whereas 4d and 4e were identified in all compartments and had the same *m*/*z* (*m*/*z* 341), but their product ion spectra differed. In the product ion spectra of 4c, 4d, and 4e no product ion with an *m*/*z* ratio of 193 (as shown for the other metabolites of 4) was obtained. These results indicate that the basic structure of 4 may have been metabolically altered. The structures of these metabolites are still being elucidated.

3.5 Transport of apple-derived flavonoids

In addition, we investigated both the transport and epithelial metabolism of the apple-derived flavonoids phloretin 14, quercetin 17, (–)-epicatechin 19, and (+)-catechin 20 (and selected glycosides), at concentrations displayed in Table 1, using the Ussing chamber model system. The only flavonoid for which transport across the T84 monolayer was observed was 14, for which final levels in the cell suspension and basolateral compartment were equivalent to 9.4 and 3.3% of the initial amount, respectively (Table 6). After

Table 5. Transepithelial transport experiments

R_t (min)	Precursor ions (*m*/*z*)	Product ions (*m*/*z*)	Compound	Apical	Basolateral	Cell associated
12.3	368.9	192.9, 174.9, 112.9	Feruloyl glucuronide 4a	+	+	+
14.0	273.0	192.9, 177.7, 149.1, 133.7, 96.9, 79.6	Feruloyl sulfate 4b	+	+	+
18.2	194.8	135.9	Hydroferulic acid 13	n.d.	+	n.d.
19.5	192.9	177.9, 148.9, 133.9	Ferulic acid 4	+	+	+
19.8	307.2	232.9, 229.2, 189.0, 133.5, 114.8, 97.8	Unknown 4c	n.d.	n.d.	+
25.7	341.3	281.1, 172.8, 158.9, 122.9, 107.9, 91.0	Unknown 4d	+	+	+
27.4	341.3	325.9, 311.1, 297.0, 282.1, 266.9, 172.8, 159.2, 123.0, 107.9, 90.8	Unknown 4e	+	+	+

Retention times (R_t), precursor ions, product ions, and distributions of metabolites of ferulic acid after incubation in the Ussing chamber with T84 monolayers for 4 h at 1000 μM.
+, detected; n.d., not detected.

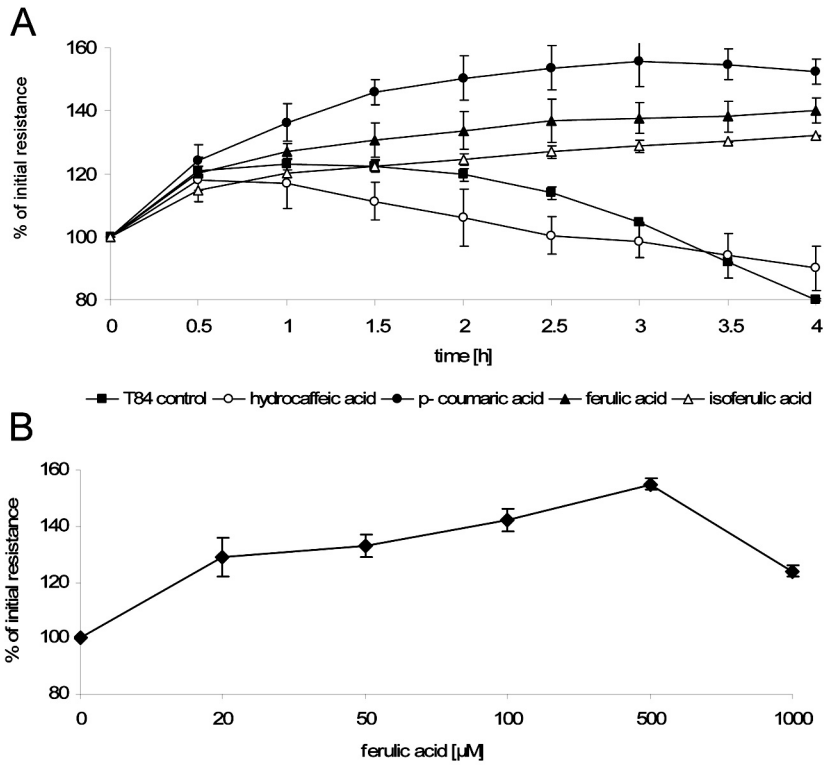


Figure 6. (A) Time- and (B) dose-dependent effects of polyphenols on the TER of T84 cells. (A) TER measurements obtained over 4 h relative to controls. (B) Effects of 20, 50, 100, 500, and 1000 μM ferulic acid on the TER of T84 cell monolayers after incubation for 4 h. Values are means ± SD obtained from three experiments. Asterisks indicate significant differences from controls: $^{*}p \leq 0.05$, $^{***}p \leq 0.001$.

incubations of phloretin 2′-*O*-glucoside 15 and phloretin 2′-*O*-xyloglucoside 16 the respective glycosides were detectable in the cell suspensions (but not on the basolateral side). Following incubation of 15 with T84 monolayers the aglycone 14 was detected in the cell suspension (at levels equivalent to 0.3 ± 0.2% of the initial amount of 15). However, when quercetin 17 was incubated with the monolayers this compound was not detected in either the apical or the basolateral compartment, but small amounts of unconjugated 17 were found in the cell suspension. Further, in the apical compartment of the Ussing chamber, two degradation products of 17, 3,4-dihydroxybenzoic acid (17a) and 2,5,7,3′,4′-pentahydroxy 3,4-flavandione (17b), were identified (see cell-free HBSS incubations, Section 3.8). Quercetin 3-*O*-rhamnoside 18, 19, and 20 were detected in the apical compartment, but not either the basolateral chamber or the cell suspension. Glucuronides of 14 and 17 were detected in trace amounts in the cell suspensions using HPLC-ESI-MS/MS (data not shown).

3.6 Influence of polyphenols on the TER of T84 monolayers

Measured TER values of confluent monolayers of T84 cells ranged from 400 and 650 Ω cm^2 in the absence of polyphenols and C10, and significant enhancement of their resist-

Table 6. Transepithelial transport experiments

Compound	Percentages (%) recovered		
	Apical	Basolateral	Cell associated
Phloretin 14	65.7 ± 0.8	3.3 ± 0.5	9.4 ± 0.1
Phloretin 2′-*O*-glucoside 15	110.5 ± 6.4	n.d.	2.9 ± 0.2
Phloretin 2′-*O*-xyloglucoside 16	103.4 ± 1.5	n.d.	1.1 ± 0.1
Quercetin 17	n.d.	n.d.	1.8 ± 0.2
Quercetin 3-*O*-rhamnoside 18	99.9 ± 6.2	n.d.	n.d.
(−)-Epicatechin 19	88.8 ± 0.9	n.d.	n.d.
(+)-Catechin 20	44.8 ± 4.8	n.d.	n.d.

Distribution of apple flavonoids in the cell suspensions and both the apical and basolateral sides of the Ussing chambers and T84 monolayer transepithelial transport model after 4 h incubation. Each value is the mean ± SD obtained from three experiments, as detailed in Section 2.
n.d., not detected.

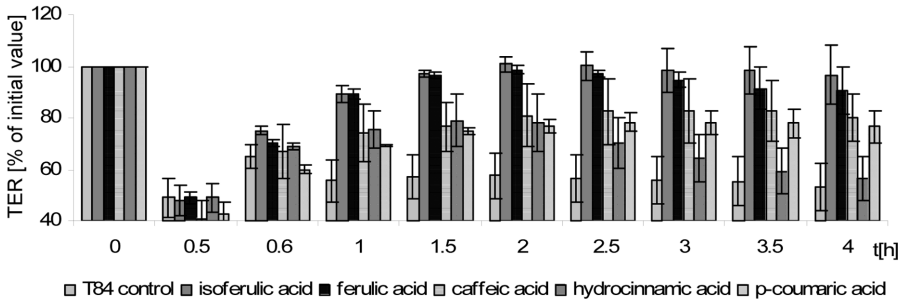


Figure 7. Effects on the TER of T84 monolayers of selected hydroxycinnamic acids (isoferulic, ferulic, caffeic, hydrocinnamic, and *p*-coumaric acid, 20 μM) added at 0.5 h, following treatment with 10 mM C10 and washing with fresh, C10-free medium. Each value is the mean ± SD obtained from three experiments.

ance was observed during their incubation in Ussing chambers for 4 h with *p*-coumaric acid 2, ferulic acid 4, isoferulic acid 5, and (more weakly) hydrocaffeic acid 12 (Fig. 6A).

Ferulic acid 4 also had dose-dependent effects on TER; 20–100 μM of this hydroxycinnamic acid increased it to 135 ± 10% of the initial resistance, whereas the effects of 1000 μM were weaker (128 ± 4% of initial resistance) at the end of the experiment. Maximum TER values were achieved with a concentration of 500 μM (155 ± 2% of initial resistance; Fig. 6B).

3.7 Influence of polyphenols on the TER of C10-treated T84 monolayers

As described in Section 2, the effects of flavonoids on T84 monolayers that had been treated with capric acid (C10) until the TER decreased to 50 ± 4% of its initial value (t = 0.5 h) then washed with HBSS buffer (which slightly increased the TER to 57% of its initial level) were also examined. TER was not further reversed in control cells, but when hydroxycinnamic acids were added (at concentrations shown in Table 1 and Figs. 1 and 2) after the C10 wash-out (t = 0.6 h) the TER of the monolayers increased up to more than 100% of its initial value (see Fig. 7).

The more polar hydroxycinnamic acids (ferulic acid 4, isoferulic acid 5, and hydroferulic acid 13) had the strongest effects. The TER of T84 monolayers incubated with *p*-coumaric acid 2 and caffeic acid 3 increased to up to about 95% of the initial value, while that of monolayers incubated with the hydroxycinnamic acid esters of D-(−)-quinic acid, 4-*p*-coumaroylquinic acid 6, 1-caffeoylquinic acid 7, and 5-caffeoylquinic acid 8 increased to 126, 133, and 104% of initial levels, respectively. TER values of T84 monolayers incubated with methyl *p*-coumarate 9, methyl caffeate 10, and hydrocinnamic acid 11 differed little from those of controls.

The effects of quercetin 17 and quercetin 3-*O*-rhamnoside 18 on the TER were in similar ranges (increasing it to *ca*. 90% of initial values), while the TER of T84 monolayers incubated with phloretin 14, phloretin 2′-*O*-glucoside 15, and phloretin 2′-*O*-xyloglucoside 16 increased to *ca*. 81, 88, and 111% of initial levels, respectively. The flavan-3-ols (−)-epicatechin 19 and (+)-catechin 20 also showed positive effects, increasing TER to *ca*. 95% of initial values.

No evidence of increased transport (mediated by enhanced permeability) of tested polyphenols was detected, *i.e.*, no additional changes in the polyphenol concentrations in the apical and basolateral compartments were observed.

3.8 Transcriptional regulation of the TJ components ZO-1, occludin, and claudin-4

To assess the effects of polyphenols (at concentrations shown in Table 1 and Figs. 1 and 2) on the expression of genes encoding the TJ components ZO-1, occludin and

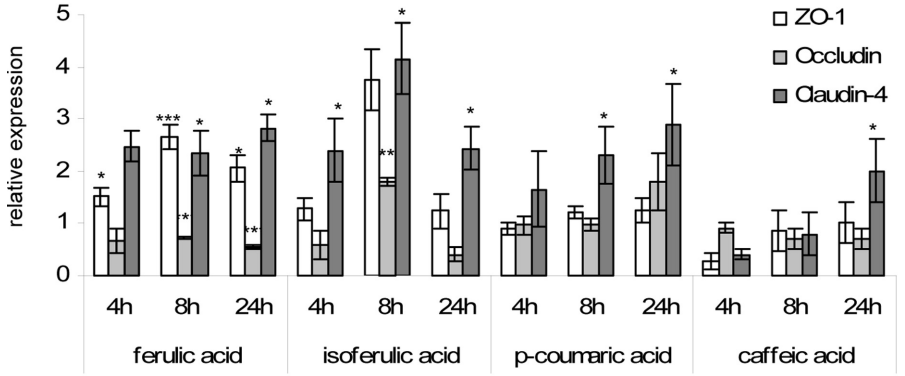


Figure 8. Expression of ZO-1, occludin, and claudin-4 mRNA in T84 monolayers after incubation with hydrocinnamic acids in the Ussing chambers. mRNA levels are normalized to those of GAPDH, and data are expressed as ~fold changes in mRNA transcript levels relative to those of T84 controls. Each value is the mean ± SD obtained from three experiments.

claudin-4, transcript levels of these genes were quantified using Taqman real-time PCR with specific primers, and normalized to GAPDH signals. The results revealed that ferulic 4 and isoferulic acid 5 induced almost 2.5- and 2.8-fold increases in ZO-1 mRNA, together with 2.8- and 4-fold increases in claudin-4 mRNA in a time-dependent manner (Fig. 8). Transcription of occludin mRNA was induced by 5 after 8 h, but in all other cases (*i.e.*, all other combinations of time and test compound) its expression level was reduced (to <1). Compound 3 induced reductions in both ZO-1 and occludin mRNA levels, and only two-fold increases in claudin-4 after 24 h, relative to control levels. By simultaneous quantification of TER it was ruled out that this rise in transcript level was confirmed by an increase of TER (see Fig. 6A).

4 Discussion

4.1 Stability of polyphenols

Determinations of the stability of the polyphenols in the cell-free HBSS incubation buffer at 37°C (Table 2) indicate that substances with two *ortho*-hydroxy groups, such as caffeic acid 3 and its derivatives or quercetin 17 are less stable than those with only one hydroxy group, such as *p*-coumaric acid 2 and its derivates or phloretin 14. Methylation of one of the hydroxy groups, as in ferulic acid 4 and isoferulic acid 5, also inhibits degradation. For the flavonoids, we showed that glycosylation of the aglycone improves the stability in HBSS. The aglycone quercetin was not stable in HBSS, whereas quercetin 3-*O*-rhamnoside was stable for 8 h. 14, which does not possess two *ortho*-hydroxy groups, was stable over 8 h, whereas its glucoside was stable for 24 h.

The possibility that H_2O_2 may have affected the stability of the polyphenols in the buffer could be excluded, since no H_2O_2 was detected in the media after incubation in the Ussing chambers (data not shown), although H_2O_2 could have been produced in the media and then volatilized and removed during the gassing of the chambers.

In incubations of 5-caffeoylquinic acid 8 with T84 cells no isomerization within 4 h was observed, although isomerization to 3- and 4-caffeoylquinic acid occurred within 4 h in the cell-free system, and isomerization in alkaline conditions (pH 8) has been previously described [36].

In our study, we observed the rapid formation of 3,4-dihydroxybenzoic acid 17a and the previously unreported formation of 2,5,7,3′,4′-pentahydroxy 3,4-flavandione 17b in HBSS cell culture media, which we attribute to the oxidative degradation of 17 under alkaline conditions. We confirmed the formation of 17b by incubation of quercetin 17 in $^{18}O_2$ enriched HBSS demonstrating the incorporation of ^{18}O into the molecule by comparison of HPLC-ESI-MS/MS product ion spectra of 17, 17b and ^{18}O-17b. Complementary studies supported these findings since (for instance) quercetin was rapidly degraded in DMEM media (data not shown). However, the formation of 2,5,7,3′,4′-pentahydroxy 3,4-flavandione from quercetin in basic (pH 8) aqueous solutions has been described by Buchner *et al*. [37], following incubation with mushroom tyrosinase [38], or oxidation with silver oxide [39]. These authors also postulate that an oxidative mechanism was involved in the cited phenomena.

Incubations of the flavan-3-ol (−)-epicatechin 19 with T84 cells resulted in the formation of flavan-3-ol quinones, whose formation has been described after incubations in Tris–HCl buffer [27], during tea fermentation processes [40], following enzymatic oxidation [41], and reactions with 1,1-diphenyl-2-picrylhydrazyl radicals [42, 43].

Other polyphenols examined were also unstable under the incubation conditions used. With the knowledge of the degradation products described above it could be excluded that these products were derived from cell metabolism.

4.2 Transcellular transport of hydroxycinnamic acids and flavonoids

The T84 cell line, derived from a human colon carcinoma, is a well-established model of differentiated human colon cells [5] that presents morphological characteristics of well-differentiated epithelial cells. T84 cell monolayers retain polarity, form intercellular TJ, and grow to confluence with the basolateral surface attached to the growth substratum and their microvillus membrane facing the

media [44]. T84 monolayers have been successfully used as intestinal secretory model systems for more than two decades in Ussing chamber experiments [45], and their use allows interactions between the epithelium and various substances, especially those related to electrolyte transport processes, to be readily investigated. The T84 cell line has been used (*inter alia*) in comparative studies of intestinal, mast cell and/or lymphocyte secretions and cell products. The effects of various compounds on epithelial paracellular and transcellular pathways have also been examined using T84 cells [45]. Differentiated polarized intestinal T84 cells form TJ, thus restricting the paracellular permeation pathway, which is the preferred route of absorption for small hydrophilic molecules.

Studying the bioavailability of polyphenols *in vitro* and in animal studies, do have to be reassessed in humans in order to approve their significance. Clinical and epidemiological studies are needed to provide definitive proofs of the positive effects of polyphenols [2].

In the study presented here, we investigated the effects of a wide range of polyphenols, including various hydroxycinnamic acids and flavonoids, on T84 cells mounted in Ussing-type chambers. Studied effects included transport and metabolism of the polyphenols and simultaneously their impact on TER of the T84 monolayers. Relatively high rates of transport across the T84 monolayers were detected for the less polar hydroxycinnamic acids cinnamic acid 1 and hydrocinnamic acid 11, since 29.0 and 24.8% of the initial concentrations were found in the basolateral compartment after 4 h incubation (Table 3). In contrast, only 2.3 and 3.2%, respectively, of the apically administered doses of ferulic acid 4 and isoferulic acid 5 (which have methoxylated ring systems) were found in the basolateral chamber, indicating that these compounds are passively transported across the monolayer, as previously reported [6, 46]. The high transportation rates of the methyl esters of caffeic acid 10 and *p*-coumaric acid 9 corroborated these findings. We found that transport rates decreased in the order 10 > 9 > 1 > 11 > 4 > 5. Transport to the basolateral side was higher for hydroxycinnamic acids methoxylated at the carboxyl group (9 and 10) than for those methoxylated at the ring (4 and 5). Transport across the T84 monolayer of 1, which has a double bond in the side chain, was higher than that of 11, which has a single bond in the side chain. For all studied hydroxycinnamic acids hydroxylation of the ring inhibited transport across the T84 monolayer at physiological concentrations, as shown for 2 and 3, but methoxylation of the carboxyl group of these substances increased transport rates, as shown for 9 and 10.

Quantification of some polyphenols in the compartments revealed slight concentrations (by up to 15%; see Tables 3, 4, and 6), which we attribute to evaporation of the solvent water during the 4 h of continuous gassing of the Ussing chambers.

Konishi *et al*. [7, 8] detected transport of 2, 3, and 8 to the basolateral side of Caco-2 monolayers. We detected no significant transport of these polyphenols across our T84 monolayers. However, in contrast to Konishi *et al*., who used initial concentrations ranging from 1 to 5 mM, we applied physiological concentrations, *i.e*., concentrations reportedly found by Kahle *et al*. [3] in ileostomy bags after subjects had consumed 1 L of cloudy apple juice, representing the concentrations reaching the colon of healthy subjects. For hydroxycinnamic acids (1, 2, 3, 4, 5, 6, 9, 10, 11, 12, 13), concentrations of 20 μM were used, while for 8 and 7 concentrations of 50 and 10 μM, respectively, were administered to the apical side of the Ussing chambers.

In Caco-2 cell line several ABC transporter proteins like MDR1, MRP2, BCRP, MCT1, and S-MCT are involved in transport of polyphenols [6, 12]. Up to now there is few evidence for their presence in T84 cells as described in Section 1.

4.3 Formation of hydroxycinnamic conjugates

No measurable metabolites of hydroxycinnamic acids were detected in the cell suspensions or on either the apical or basolateral side of the chambers. Consequently, ferulic acid 4 was incubated at 20, 50, 100, 500, and 1000 μM concentrations, according to Poquet *et al*., [6] in order to determine the ability of T84 cells to form conjugates such as glucuronides and sulfates of hydroxycinnamic acids, as described for Caco-2 cells. Our results (Table 4) show that 4 was successfully transported across the T84 monolayers. These findings are consistent with data presented by Poquet *et al*. [6]. Conjugates such as glucuronides and sulfates were detected by HPLC-ESI-MS/MS when high concentrations (100 – 1000 μM) of 4 were supplied (Table 5), but no conjugates were detected (*i.e*., their levels were below the LOD) when physiological concentrations (20 μM) were supplied. The formation of conjugates such as glucuronides and sulfates in experiments with Caco-2 as intestinal cell models has been previously described for ferulic acid [6], 5-caffeoylquinic acid, caffeic acid [8], *p*-coumaric acid [7], (−)-epicatechin [11], and methyl hydroxycinnamates [9].

4.4 Transcellular transport of the flavonoids quercetin and phloretin

Following incubation of the flavonoids quercetin 17 and phloretin 14 at physiological concentrations (10 and 20 μM, respectively, Table 3) with T84 monolayers the test compound was detected in the cell suspensions, in both cases, together with traces of glucuronides. The formation of several quercetin *O*-glucuronides in both *in vitro* [47] and *in vivo* [48 – 50], experiments has been reported, and phloretin 2′-*O*-glucuronide was recently identified in ileostomy bags after subjects had consumed cloudy apple juice [4]. In our study, the position of the glucuronidation could not be identified since the samples were too small. For quercetin 3-*O*-rhamnoside 18, phloretin 2′-*O*-glucoside 15, and

phloretin 2′-*O*-xyloglucoside 16 at physiological concentrations no transport across the T84 monolayer was observed, in agreement with findings for other glycosides [51]. Kobayashi *et al*. [52] incubated Caco-2 cells with 500 μM of the flavanone glycoside hesperedin (hesperitin 7-*O*-rhamnoglucoside) and found 0.58% of the initial amount in the basolateral chamber. Due to the low, physiological concentrations of the glycosides used in our study (10 and 20 μM, respectively) such amounts would have been below the detection limits [51]. In contrast, physiological concentrations (10 μM) of the aglycon hesperitin were incubated by Brand *et al*. in Caco-2 cells. Transport of the aglycon to the basolateral chamber was 4% of the initial concentration and high conjugation rates of hesperitin could be observed due to the nature of the aglycon [12].

4.5 Influence of polyphenols on TER

Maintenance of the colon barrier function strongly depends on intact TJ, which prevent the invasion of potential pathogens into the underlying tissues by paracellular diffusion [53]. The components of TJ include proteins spanning the cytoplasmic membrane (*e.g*., occludin and claudin-4) and cytoplasmic proteins that link these membrane proteins to the cytoskeleton (*e.g*., ZO-1). Occludin plays a major role in the regulation of TJ barrier function and is recruited by ZO-1 to cell junctions [54, 55].

In our study, we examined the influence of polyphenols on the TER of monolayers that had, and had not, been damaged by capric acid (C10), and their influence on TJ-related genes at physiological concentrations. The weakening of the gut barrier is of high clinical interest in intensive care medicine in the context of sepsis and trauma.

Some of the tested polyphenols induced increases in the TER in comparison to the controls (Fig. 7). These findings are in agreement with results published by Ohno *et al*. [56], who found increases in the TER of Caco-2 monolayers after incubating them for 4 h with quercetin glycosides at 80 μM. However, other authors detected no significant effects of raspberry extracts containing various anthocyanins, ellagitannins, and hydroxycinnamic acid derivatives on the TER of Caco-2 monolayers [57].

4.6 Influence of polyphenols on repair of epithelial injury

In studies of epithelial injury and repair in experimental colitis, several noxious agents have been used, including deoxycholate, alcohol, acetic acid, and hydrochloric acid. While these models do not reflect all aspects of human colonic disease, they do provide information about mechanisms whereby the colonic epithelium can recover from injury [58 – 61]. We chose the sodium salt of capric acid as a noxious agent for the T84 monolayers mounted in Ussing chambers, which affects TJ by reducing ATP formation, thereby inducing contractions of the perijunctional actomyosin rings [29] and thus increasing paracellular permeability. At a concentration of 10 mM C10 dilates TJ and increases paracellular fluxes across both Caco-2 monolayers and rat ileum mounted in Ussing chambers [62, 63]. The technique applied here to measure postinjury epithelial recovery has been previously used [61]. We ascertained that polyphenols can induce full recovery of the TER of C10-treated T84 monolayers in which the TER has been reduced by 50%, while the effect was not totally reversed in control cells. However, no increase in the fluxes of the tested polyphenols was detected after apical injury with C10 (data not shown), conflicting with results of earlier experiments in which Caco-2 monolayers were treated with the actin-perturbing drug cytochalasin D from the apical side to obtain monolayers with varying TERs [64]. Amounts of hesperidin (hesperitin 7-*O*-rhamnoglucoside) [52], tea catechins [65], 5-caffeoylquinic acid, and caffeic acid [8] transported have been found to be higher across monolayers with low TER than across those with high TER. However, in the cited experiments the noxious agent remained in contact with the monolayers, whereas in our experimental setup the treatment with C10 to decrease the paracellular permeability was followed by a wash-out with C10-free buffer. The TER of the control monolayer did not increase after the wash-out, showing that the lowering of the TER was not reversible simply by the removal of C10 (wash-out).

4.7 Influence of polyphenols on the formation of TJs

The colonic mucosal surface is constantly exposed to potential pathogens, TJ constitute the main paracellular barrier in epi- and endothelia to these pathogens, and several intestinal diseases have been associated with disorders of the TJ [66, 67]. The structures of TJs have been mainly investigated at the protein level, particularly with respect to subcellular localization. In this study, we focused on the regulation of the TJ constituents ZO-1, occludin, and claudin-4 at the transcript level using Taqman probes [68] to investigate the influence of various polyphenols in order to fortify our TER-results. Our findings on modulatory effects of polyphenols on TJ proteins (Fig. 8) add to the large number of beneficial effects reported for dietary flavonoids, including suppression of carcinogenesis and prevention of cardiovascular disease [69, 70]. The direct induction of the expression of a "sealing" TJ protein by these polyphenols is a novel finding. The TER was increased, to up to 155% of its initial value, in the presence of the optimal concentration of 4 (500 μM), and it was not repressed even by a very high concentration (1 mM). Thus, the increased transport was not due to a defective monolayer. Furthermore, transcription of the TJ elements ZO-1, occludin, and claudin-4 was also stimulated by some of the tested polyphenols (data not shown). Overexpression of claudin-4 in epithelial cells has

been shown to increase TER and reduce their Na^+ permeability, but to have no effects on their permeability toward certain other substance, *e.g.*, mannitol [71]. This pattern of alterations is consistent with the changes seen here. The functionality of TJ and their components has been addressed in several studies, but regulation of the transcription of the genes involved in responses to exogenous factors has been rarely investigated in the past [72, 73].

Clinical interest is growing in therapies based on supplementary trace elements and vitamins. For example, glutamine has been demonstrated to improve intestinal barrier functions in highly stressed patients [74] and to prevent parenteral nutrition-induced increases in intestinal permeability [75]. In addition, the effects of SCFAs on paracellular permeability have been investigated, and butyrate, acetate, and propionate have all been found to induce concentration-dependent reductions in paracellular permeability *in vitro*, in the Caco-2 colonic epithelium model, causing mechanical distension, promoting differentiation, and improving barrier function [76]. Notably, in the context of this study, some of the investigated polyphenols act as direct intestinal protective agents by improving the intestinal barrier function.

4.8 Conclusion

Our results provide confirmation that T84 cells, like the Caco-2 cell line, could be used as model systems in the future to simulate the intestinal mucosa. The transport of some polyphenols across T84 monolayers was observed and conjugates were generated. Further, our studies showed that polyphenols are able to increase the TER of both untreated and C10-treated T84 monolayers. These findings not only advance our understanding of the regulation of the paracellular intestinal barrier but may also facilitate the future design and refinement of therapeutic barrier-enhancing strategies during treatments of conditions associated with intestinal inflammation, *e.g.*, inflammatory bowel disease and postinfectious irritable bowel syndrome.

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