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Site-specific accumulation of the cancer preventive dietary polyphenol ellagic acid in epithelial cells of the aerodigestive tract

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Abstract

Ellagic acid (EA), a polyphenol present in berries, has been demonstrated to prevent oesophageal and colon cancer in animals. To better understand the site-specificity of these effects, we studied the accumulation and transport of [^{14}C]EA in rat aerodigestive epithelial cells in-vivo and in cultured human cells. When [^{14}C]EA was administered to rats by gavage, a high content of EA was found in the oesophagus and small intestine at 0.5 h after oral administration and in the colon at 12 h, with very low amounts in plasma and peripheral tissues. Studies in human intestinal Caco-2 and human oesophageal HET-1A cells found very limited transcellular transport (Caco-2) of EA but high accumulation (Caco-2 and HET-1A) in the cells. In more detailed studies in the Caco-2 cells, accumulation of EA displayed ATP- and Na^+-dependency. Multiple interventions permitted the exclusion of a number of transporters as mediators of this uptake. A dramatically reduced transport of EA at low pH (5.5) compared with high pH (7.4) suggested an important role for the negative charge of EA. This was supported by the organic anion transport inhibitors 4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid and bromosulfophthalein. The latter produced as much as 78% inhibition at the 100 μM concentration. Finally, Caco-2 cells were shown to express organic anion transporter 4 (OAT4) mRNA, as was the human large intestine. EA appears to be accumulated along the aerodigestive tract using OAT-like transporters, one of which might be OAT4.

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Introduction

Diets rich in fruits and vegetables are well known to contain a variety of chemicals that can affect the carcinogenic process in many ways (Middleton et al 2000; Key et al 2002). Ellagic acid (EA, Figure 1), one of these chemicals, is found in various fruits and nuts (Lei et al 2001; Mattila & Kumpulainen 2002). EA is thought to have a role in eliciting the chemopreventive effect of different berries, as seen in various animal models specific for oesophageal and colon cancers (Stoner et al 1999; Carlton et al 2001; Kresty et al 2001). In fact, EA directly has displayed chemopreventive properties in the same animal models against several classes of chemical carcinogens (Lesca 1983; Dixit et al 1985; Mandal & Stoner 1990; Rao et al 1995; Stoner & Gupta 2001).

There is little information on the disposition of EA after oral administration, particularly in man. Teel & Martin (1988) reported poor bioavailability of EA in mice as shown by low levels in blood and bile at 2 and 24 h post-administration. They found most absorbed radioactivity excreted in the urine within the first 2 h. Boukharta et al (1992) also observed poor bioavailability of EA in A/J mice after oral administration. Smart et al (1986) found little or no EA in blood or several peripheral tissues of CD-1 mice after administration in the diet for one week. However, these studies did not focus on the ability of EA to accumulate in aerodigestive epithelial tissues.

In a previous study, using human intestinal Caco-2 cells, we found very limited transcellular transport of EA (Whitley et al 2003), consistent with the very low absorption in the animal studies. However, these epithelial cells demonstrated a remarkable accumulation of EA (Whitley et al 2003). A potential explanation for the site-specific cancer chemopreventive effects of EA may be site-specific delivery through specific transport. In a parallel

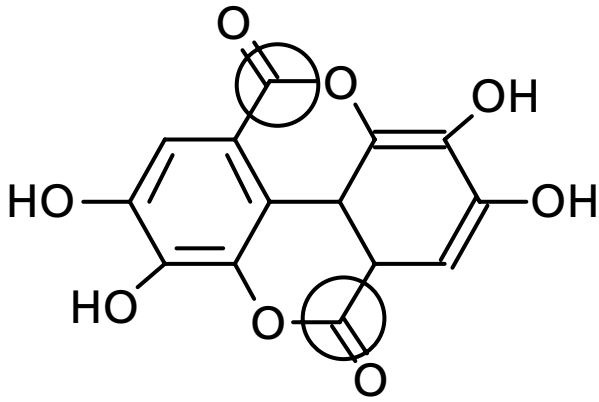

Figure 1 Chemical structure of ellagic acid (EA). The sites of [^{14}C] labelling are circled.

study using a *Xenopus laevis* oocyte expression assay, we found that EA can in fact be transported by the human organic anion transporters 1 and 4 (hOAT1 and hOAT4) in this model system (Whitley et al 2005).

This study was therefore focused on identification of membrane transporters that may have high affinity for EA by investigating a wide spectrum of possible transporters which may be present in the human gastrointestinal system. Such transporters may also play a role in the absorption of many small organic anionic therapeutics across the intestine. These studies were carried out in-vivo in rat and in human intestinal Caco-2 and oesophageal HET-1A cells.

Materials and Methods

Materials

[^{14}C]Ellagic acid (Figure 1) was synthesized with a specific activity of 20 mCi mmol^{-1} at the Ohio State University Comprehensive Cancer Center (Zeng et al 1991) and was a kind gift from Dr Gary Stoner, Ohio State. TS-2 tissue solubilizer was obtained from Research Products International (Mt Prospect, IL). Normal human cDNA libraries (kidney – 68-year-old female, oesophagus – 28-year-old male, small intestine – 26-year-old male, large intestine – 58-year-old female) were purchased from BioChain Institute, Inc. (Hayward, CA). Fetal bovine serum was purchased from Atlas (Ft Collins, CO) and HBSS and other cell culture medium components were obtained from Cellgro, Mediatech (Herndon, VA). Other chemicals were purchased from Sigma-Aldrich (St Louis, MO) or Fisher Scientific.

In-vivo disposition

The Institutional Animal Care and Use Committee of the Medical University of South Carolina approved the study. Male Fischer 344 rats, 200–250 g, were acclimatized to a 12-h light–dark cycle for 1 week before use. Food was removed 12 h before administration of [^{14}C]EA. Both oral and intravenous administrations consisted of [^{14}C]EA (0.19 mg) in 0.5 mL polyethylene glycol 400–ethanol (9:1). The oral dose was administered via gavage. The intravenous dosing group was anaesthetized with isoflurane and [^{14}C]EA was administered through a cannulated saphenous leg vein. The rats were sacrificed by CO_2 asphyxiation at 0.5 or 12 h after EA administration (6 rats/group/time point, except where noted). Blood was immediately collected by heart puncture in the presence of EDTA to prevent clotting. The samples were centrifuged for 3 min at 16 000 rev min^{-1} and the plasma was removed and frozen. The oesophagus, small intestine, and large intestine were excised and rinsed thoroughly with 3 volumes of cold saline. The lungs, liver, and kidneys were also collected. All tissues were frozen in liquid nitrogen and kept at −20°C. Portions (400 mg) of each tissue were homogenized with a Polytron homogenizer in ice-cold saline. The samples were analysed for total [^{14}C]EA in each tissue after solubilization in TS-2 tissue solubilizer with shaking overnight. Hydrogen peroxide and scintillation cocktail were added for bleaching and scintillation spectroscopy, respectively. Plasma was analysed by addition of scintillation cocktail and scintillation spectroscopy.

Cell culture

Normal human oesophageal epithelial HET-1A cells (Stoner et al 1991) were cultured at 37°C in an atmosphere of 5% CO_2 and 90% relative humidity in Dulbecco's Minimum Essential Medium with high glucose (4.5 g L^{-1}) and 2% fetal bovine serum, 1 mM sodium pyruvate, 2 mM L-glutamine, 5 μg mL^{-1} insulin, 5 μg mL^{-1} transferrin, 0.01 μg mL^{-1} hydrocortisone and 0.01 μg mL^{-1} cholera toxin, as previously described (Orlando et al 2002). Human intestinal epithelial Caco-2 cells (ATCC, Rockville, MD) were cultured in Eagle's Minimum Essential Medium with 10% fetal bovine serum, nonessential amino acids and penicillin/streptomycin in a 37°C humidified incubator with 5% CO_2, as previously described (Walgren et al 1998). Cell uptake experiments were performed with confluent monolayers, typically 7–10 days after seeding.

Cellular accumulation of [^{14}C]EA

Caco-2 and HET-1A cells were seeded in plastic 6-well plates. Caco-2 cells were used at passage 70–80 and HET-1A cells were used at passage 35–43. Experiments were done in Hanks' balanced salt solution (HBSS) with 25 mM HEPES (pH 7.4) without phenol red, unless otherwise noted. The cells were washed twice for 30 min with warm buffer followed by the addition of 10 μM [^{14}C]EA in buffer. Where applicable, the transport inhibitors were included in the second 30-min wash and the uptake medium, except for bromosulfophthalein (BSP), which was only included in the uptake medium. Uptake ± transport inhibitors (4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid (DIDS), nystatin, BSP, taurochenodeoxycholate (TCDC), cholate, quercetin, glucose, ascorbate and phloridzin) was measured for 2 min. All experiments were stopped by the addition of 5 volumes of ice-cold HBSS. The cells were then washed 3 times with 1 mL of ice-cold HBSS. The cells were digested with sodium hydroxide and analysed for [^{14}C]EA content by liquid scintillation spectroscopy and for protein content (Lowry et al 1951).

In experiments designed to examine whether the apical membrane transport is Na^+-dependent, phosphate-buffered saline (PBS, 140 mM Na^+) was replaced with sodium-free PBS with equimolar choline chloride (140 mM). Nystatin, an ionophore for monovalent cations (Vemuri et al 1989), was also used to examine sodium dependence. In experiments designed to examine whether the apical membrane transport is ATP-dependent, the cells were depleted of ATP with 10 mM sodium azide and 10 mM 2-deoxy-D-glucose (Nagasawa et al 2002) in PBS before the addition of [^{14}C]EA. In experiments designed to examine whether the apical membrane transport is pH-dependent, the second wash and uptake buffer was buffered to the appropriate pH before use. In experiments designed to examine whether the sodium-dependent glucose transporter 1 (SGLT1) is involved, glucose (30 mM) and phloridzin (0.5 mM) were used as inhibitors (Toggenburger et al 1982). In experiments designed to examine whether the sodium-dependent vitamin C transporter (SVCT) is involved, quercetin (50 μM) and ascorbate (0.5–1.5 uM) were used as inhibitors (Song et al 2002). In experiments designed to examine whether the apical sodium-dependent bile acid transporter (ASBT) is involved, TCDC (10–200 μM) and cholate (10–200 μM) were used as competitive inhibitors (Craddock et al 1998). In experiments designed to examine whether the OATs or organic anion-transporting polypeptides (OATPs) are involved, DIDS (0.25–1 mM), a general anion transport inhibitor (Walters et al 2000), and BSP (10–500 μM) were used as inhibitors (Saito et al 1996). Nystatin and DIDS were dissolved in DMSO (final concentration < 0.1%). Accumulation factors were determined for Caco-2 cells using 3.49 μL (mg protein)$^{-1}$ (Burnham & Fondacaro 1989) and for HET-1A cells using 1.702 pl/cell (Orlando et al. 2002) and a correlation between mg protein and cell number (data not shown).

RT-PCR analysis

Total RNA was isolated from confluent, 7 days post-confluent and 14 days post-confluent Caco-2 and HET-1A cells using a guanidine isothiocyanate–phenol–chloroform extraction method according to the manufacturer's instructions (TRIZOL Reagent; Invitrogen, Carlsbad, CA). The isolated RNA was treated with DNAse I to remove any potential contaminating genomic DNA. The expression profile of hOAT1 and hOAT4 was examined by reverse transcription polymerase chain reaction (RT-PCR) in cell lines and by PCR in human cDNA libraries. cDNA was made using M-MLV reverse transcriptase following the manufacturer's instructions (Promega, Madison, WI) and PCR was performed using Taq PCR Master Mix Kit (Qiagen, Valencia, CA). Cycle parameters were: denaturing at 94°C for 15 min; followed by 35 cycles of 94°C, 60°C and 72°C for 20 s each. Plasmids containing the corresponding OATs were used as positive controls. For cDNA libraries, human kidney cDNA was used as a positive control. The following specific primers were used for PCR: 5′-gca ccg atg gct cga tct at-3′ forward and 5′-tgg ccc agg ctg tag aca ta-3′ reverse for hOAT1; 5′-ttg cgc tcc ctg aca cta tcc-3′ forward and 5′-cag tct ctg gtt ggc gtg gct-3′ reverse for hOAT4; 5′-atg ctg gcg ctg agt acg tc-3′ forward and 5′-gcc agt gag ctt ccc gtt ca-3′ reverse for hGAPDH. As a second control against potential amplification from contaminating genomic DNA, each primer in a pair is targeted to different exons such that amplification products from genomic DNA would also include intronic sequences resulting in substantially different product sizes from cDNA vs genomic DNA (i.e., hOAT1, 388 bp vs 2440 bp and hOAT4, 443 bp vs 1563 bp). PCR products were run on a 1.5% agarose gel, stained with ethidium bromide, and visualized.

Statistics

Data are expressed as means ± s.e. Statistical differences between two treatments were determined using unpaired Student's *t*-test. Differences were considered significant when $P \leq 0.05$. For comparison of four or more treatments, one-way analysis of variance with the Bonferroni post-test or the non-parametric Mann–Whitney *U*-test or Kruskal–Wallis test with the Dunn's post test was used.

Results

In-vivo disposition

The purpose of the in-vivo study in the rat was to determine the ability of EA to accumulate at sites in the aerodigestive tract compared with blood and peripheral tissues after oral dosing. Data were compared with those obtained after administering the same dose intravenously. The early time point, 0.5 h, was chosen as this had been shown to be the t_{max} value for plasma concentration of total EA in a previous study (Teel & Martin 1988). Tissues were thoroughly washed, in particular those from the aerodigestive tract, before analysis.

The distribution of radioactive EA in plasma and aerodigestive and peripheral tissues at 0.5 and 12 h after [^{14}C]EA administration is shown in Figure 2. Compared with the same dose given intravenously, the plasma concentration of EA after oral administration was 150-fold lower at 0.5 h and 15-fold lower at 12 h. The highest tissue levels of EA at 0.5 h after the oral dose were in the oesophagus and small intestine (Figure 2A), with very low levels in peripheral tissue (i.e. liver, lung and kidney), corresponding well to the low plasma levels. At 12 h after oral administration, the levels of EA in the large intestine were by far the highest (Figure 2B). As expected, the peripheral tissue EA content was very high after the intravenous dose. Interestingly, the highest level of EA radioactivity at 0.5 h after the intravenous dose was in the small intestine, suggesting some type of blood-to-enterocyte active transport system for EA. Also, a relatively high accumulation of EA in the kidneys is worth noting.

Epithelial cell accumulation

The accumulation of 10 μM [^{14}C]EA in Caco-2 and HET-1A cells is shown in Figure 3, indicating a rapid initial uptake during the first 2 min. In HET-1A cells the accumulation factor (cell-to-medium concentration ratio) was 22 ± 4 at 15 min, whereas this was considerably higher (i.e. 32 ± 2) in the Caco-2 cells at this time. Interestingly, HET-1A cells, as compared

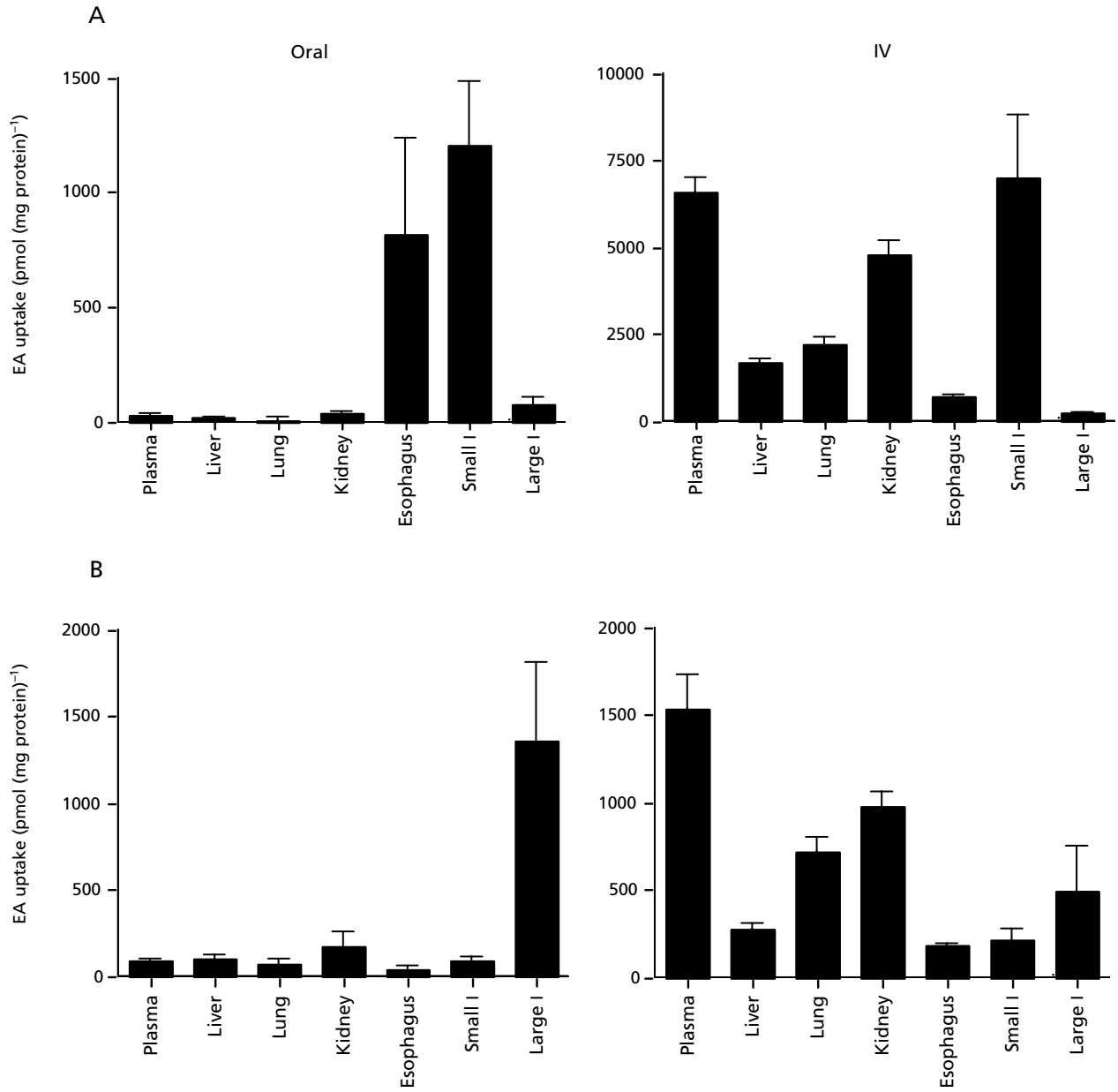

Figure 2 Distribution of total [^{14}C]EA radioactivity in plasma, aerodigestive and peripheral tissues at 0.5 h (A) and 12 h (B) after oral (left panels) and intravenous administration (right panels) of a single 0.19 mg dose of [^{14}C]EA to F-344 rats; n = 6 for all tissues except for the oesophagus where n = 4. The EA concentrations were significantly lower after oral compared with intravenous administration in plasma and all tissues except the oesophagus at the 0.5-h time-point and the large intestine at the 12-h time-point ($P < 0.005$ to $P < 0.05$, Mann–Whitney U-test).

with Caco-2 cells, contain a much larger volume per mg protein.

In attempts to identify potential apical absorptive transporters involved in EA epithelial cell uptake, 1–2 min accumulation experiments with [^{14}C]EA (i.e., initial velocity conditions as determined from Figure 3) were carried out using the Caco-2 cells in the presence and absence of transporter-modulating conditions. The uptake of [^{14}C]EA in the absence of sodium was significantly less than that observed in the presence of sodium, 363 ± 29 pmol (mg protein)$^{-1}$ versus 998 ± 127 pmol (mg protein)$^{-1}$, respectively (Figure 4). A similar reduction in [^{14}C]EA accumulation was observed in the presence of 50 μM nystatin, an ionophore for monovalent cations (Vemuri et al 1989; Walgren et al 2000) used to uncouple the sodium gradient, 536 ± 73 pmol (mg protein)$^{-1}$ versus 1132 ± 40 pmol (mg protein)$^{-1}$, respectively (Figure 4). Moreover, in ATP-depleted Caco-2 cells (Nagasawa et al 2002), [^{14}C]EA accumulation was inhibited by 45 ± 13% (data not shown). When examining the potential involvement of a pH-dependent transporter for the accumulation of

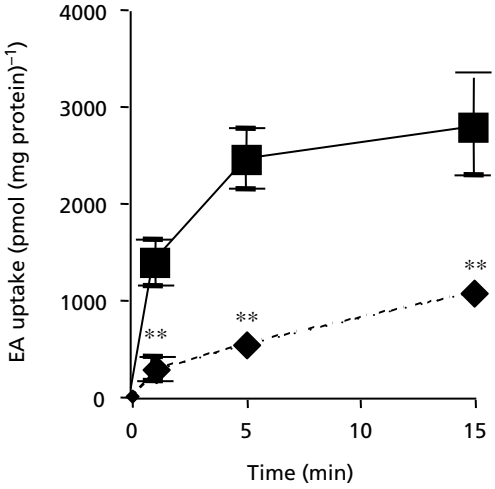

Figure 3 Time-course for the accumulation of 10 μM [^{14}C]EA in confluent Caco-2 (diamond) and HET-1A (square) cells. $^{**}P < 0.01$, vs the corresponding time-point in the HET-1A cells (Mann–Whitney *U*-test; n ≥ 3).

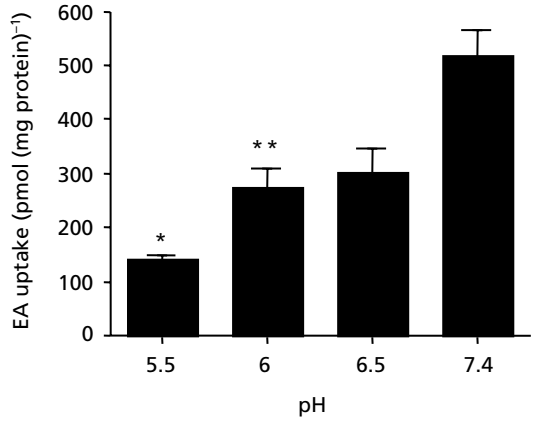

Figure 5 Effect of pH on cellular accumulation of EA in confluent Caco-2 cell monolayers. Cells were incubated for 1 min with 10 μM [^{14}C]EA at different buffer pH. $^{*}P < 0.05$, $^{**}P < 0.01$, vs pH 7.4 (Kruskal–Wallis non-parametric multiple comparisons test with Dunn's post-test; n = 6–18).

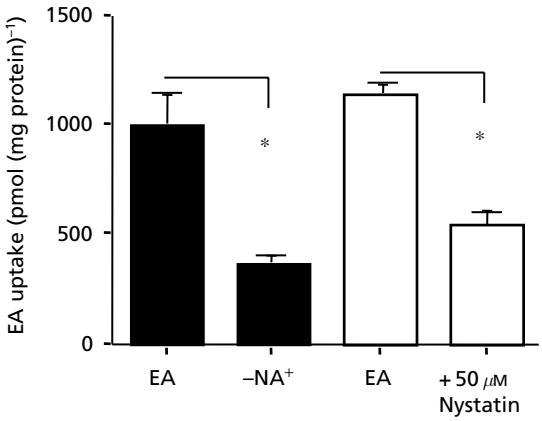

Figure 4 Effect of sodium and nystatin on cellular accumulation of EA in confluent Caco-2 cell monolayers. Cells were incubated for 1 min with 10 μM [^{14}C]EA in the presence of a normal sodium buffer (EA), a sodium-free buffer (–Na^{+}), or, in separate experiments, in the presence of 50 μM nystatin in a normal sodium buffer. $^{*}P < 0.05$ vs control (Student's *t*-test; n ≥ 9).

[^{14}C]EA, a dramatically reduced cell accumulation was observed when pH was lowered stepwise from 7.4 to 5.5 (Figure 5).

We had thus established that the absorptive transport of EA in Caco-2 cells was both ATP- and sodium-dependent. Caco-2 cells or intestinal epithelial cells express several sodium and ATP-dependent transporters on their apical membrane (e.g. the glucose transporter SGLT1 (Blais et al 1987), the ascorbate transporters SVCT1 and SVCT2 (Maulen et al 2003) and the bile acid transporter ASBT (Craddock et al 1998)). However, glucose (30 mM) and phloridzin (0.5 mM), inhibitors of SGLT1 (Toggenburger et al 1982), had no effect on the uptake of EA in the Caco-2 cells. In experiments designed to examine if SVCT 1 or 2 were involved, quercetin (50 μM) and ascorbate (0.5–1.5 mM), SVCT inhibitors (Song et al 2002), had no effect on EA uptake. The ASBT inhibitors TCDC (10–200 μM) and cholate (10–200 μM) (Craddock et al 1998) also had no effect on EA uptake in the Caco-2 cells. Thus, we could not find evidence for the participation of either SGLT1, SVCT1/2 or ASBT in EA transport, even though EA has some structural similarity to substrates of all of these three transporters. Our continued studies focused on other, less characterized, sodium-dependent transporters.

EA has acidic properties, in fact it is a lactone of a dicarboxylic acid, with the lowest pK_a value of around 6.3 (Priyadarsini et al 2002) and, as clearly shown above, the accumulation decreased with lowered pH (Figure 5), suggesting the involvement of a negative charge in transport. Therefore, we examined the potential involvement of organic anion transporters in Caco-2 cells. The general anion transport inhibitor DIDS (Kullak-Ublick et al 1994; Saito et al 1996; Masuda et al 1999) inhibited [^{14}C]EA accumulation in Caco-2 cells at 1 mM by 65 ± 5% (data not shown). However, the better accepted organic anion transporter inhibitor BSP (Saito et al 1996) inhibited [^{14}C]EA accumulation in Caco-2 cells in a dose-dependent manner (Figure 6), with 78 ± 1% inhibition at 100 μM BSP.

Although it is clear that metabolism of EA occurs in-vivo, only limited observations are available. As with other dietary polyphenols (Walle 2004), glucuronidation and sulfation are likely to occur, as already suggested (Smart et al 1986; Teel & Martin 1988). This might have affected our data in-vivo in the rat somewhat but not the transport experiments, which were carried out using initial velocity conditions, when metabolism would be expected to be minimal.

OAT expression

As the findings of decreased transport of EA by the organic anion transport inhibitors DIDS and BSP, as well as pH

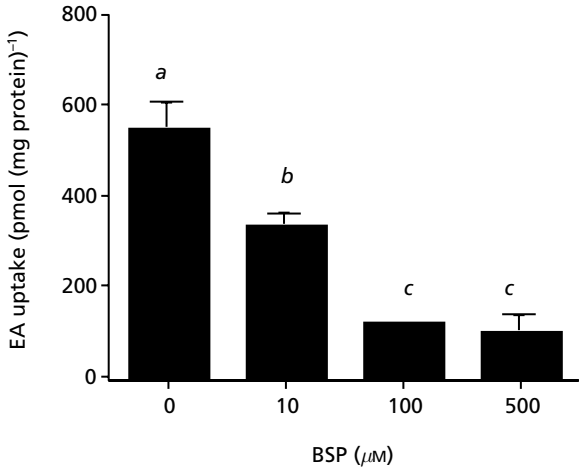

Figure 6 Effect of an anion transport inhibitor on cellular accumulation of EA in confluent Caco-2 cell monolayers. Cells were incubated for 2 min with 10 μM [^{14}C]EA in the presence of varying concentrations of BSP. Significant differences between treatments are represented by different letters, $P < 0.05$ or better; analysis of variance with Bonferroni multiple comparisons test; n = 3.

dependence, indicated the possible involvement of one or more OAT isoforms in the uptake of EA, a parallel study was undertaken, using the *X. laevis* oocyte expression assay. Oocytes were injected with various OAT cRNAs to express the corresponding membrane proteins and exposed to EA or prototype OAT substrates. EA was found to be a substrate for the human OAT forms hOAT1 and hOAT4, but not hOAT3 (Whitley et al 2005). We thus examined the expression of hOAT1 and hOAT4 in Caco-2 and HET-1A cells. Confluent Caco-2 cells, but not HET-1A cells, clearly expressed hOAT4 mRNA (Figure 7A). Interestingly, we consistently detected faint mRNA expression of hOAT4 in the colon cDNA library (Figure 7B), with mRNAs of both hOAT1 and hOAT4, as expected, in the kidney library.

Discussion

The results from the in-vivo rat study confirmed those of previous animal studies (Smart et al 1986; Teel & Martin 1988; Boukharta et al 1992), that absorption of EA from the gastrointestinal tract is very poor. Thus, the plasma concentrations after the oral dose were 50-fold less than the concentrations obtained when the same dose was given intravenously. On the other hand, after the oral dose there was a high accumulation of EA in the oesophagus and small intestine at 0.5 h, not previously observed. The high accumulation in the oesophagus may have been due to reflux or could possibly to some extent be due to removal of the gavage apparatus from the stomach into which the oral dose was administered. The high accumulation in the small intestine could be due to a site-specific accumulation of EA, maybe via a selective transporter. At 12 h, there was high accumulation of EA in the large intestine. This must have been due to the concentrating effect of enteric recirculation of EA, similar to previous reports for the flavonoid chrysin (Walle et al 1999, 2001) and genistein (Chen et al 2003). For chrysin, this type of recirculation resulted in very high accumulation in the colon (Walle et al 2001). These observations taken together indicate that the oesophagus and intestine are target tissues for cancer chemoprevention by EA, which indeed appears to be the case (Lesca 1983; Mandal & Stoner 1990; Stoner et al 1999; Carlton et al 2001; Kresty et al 2001; Stoner & Gupta 2001).

Only intravenous administration of EA provided significant distribution into peripheral tissues, such as the liver, lungs and kidneys. The relatively low EA content in the liver and the lungs as compared with plasma is worth noting. The higher content in the kidneys is interesting since renal elimination is the determinant of total body elimination for EA (Teel & Martin 1988). The high EA content in the small intestine already at 0.5 h after the intravenous dose was unexpected. This could suggest a blood to intestinal epithelial cell transporter, although no absorptive basolateral transporters have been identified in the intestine.

Based on the rat study we examined the apical accumulation of EA by two human cell lines (i.e., oesophageal HET-1A and intestinal Caco-2), determined under the same conditions. Uptake occurred in both with very high accumulation factors leading to high intracellular concentrations of EA (i.e., 224 μM in HET-1A cells and 323 μM in Caco-2 cells at an incubation concentration of only 10 μM). The high accumulation of EA was similar to a previous observation in Caco-2 cells (Whitley et al 2003).

For examination of potential apical absorptive transporters for EA we selected the Caco-2 cells, a more established cell model system. Our initial observations demonstrated that Caco-2 cell accumulation of EA was ATP- and Na^+-dependent, narrowing the list of potential transporters. While EA has some structural features consistent with it being a substrate for the known apical intestinal ATP- and Na^+-dependent transporters SGLT1, SVCT1 and 2 and ASBT, no experimental evidence supporting the involvement of these specific transporters was obtained. This was based on the lack of inhibition of Caco-2 cell uptake of EA by glucose and phloridzin (SGLT1 inhibitors), by ascorbate and quercetin (SVCT inhibitors) and by TCDC and cholate (ASBT inhibitors).

A critically important observation for our further studies was the finding of a dramatic impairment of EA transport at lower pH values, suggesting the involvement of organic anion transporters, leading to our focus on the OAT and OATP families of transporters. Also consistent with the Na^+ dependence, the mechanism of transport via the OATs has been identified to be a tertiary active system (Sweet et al 1997, 2003; Cihlar et al 1999; Ekaratanawong et al 2004), indirectly coupled to the Na^+ gradient. More specifically, an inwardly directed Na^+ gradient is established by the Na^+K^+-ATPase. The Na^+ gradient then drives absorption of dicarboxylates, including α-ketoglutarate, via an Na^+/dicarboxylate cotransporter, thus increasing the already high intracellular concentration of α-ketoglutarate maintained by metabolic activity. α-Ketoglutarate then exchanges with extracellular organic anions via the OATs. The mechanism of transport via the OATPs is not clearly defined, although it is sodium independent (Hagenbuch & Meier 2003), ruling out OATP

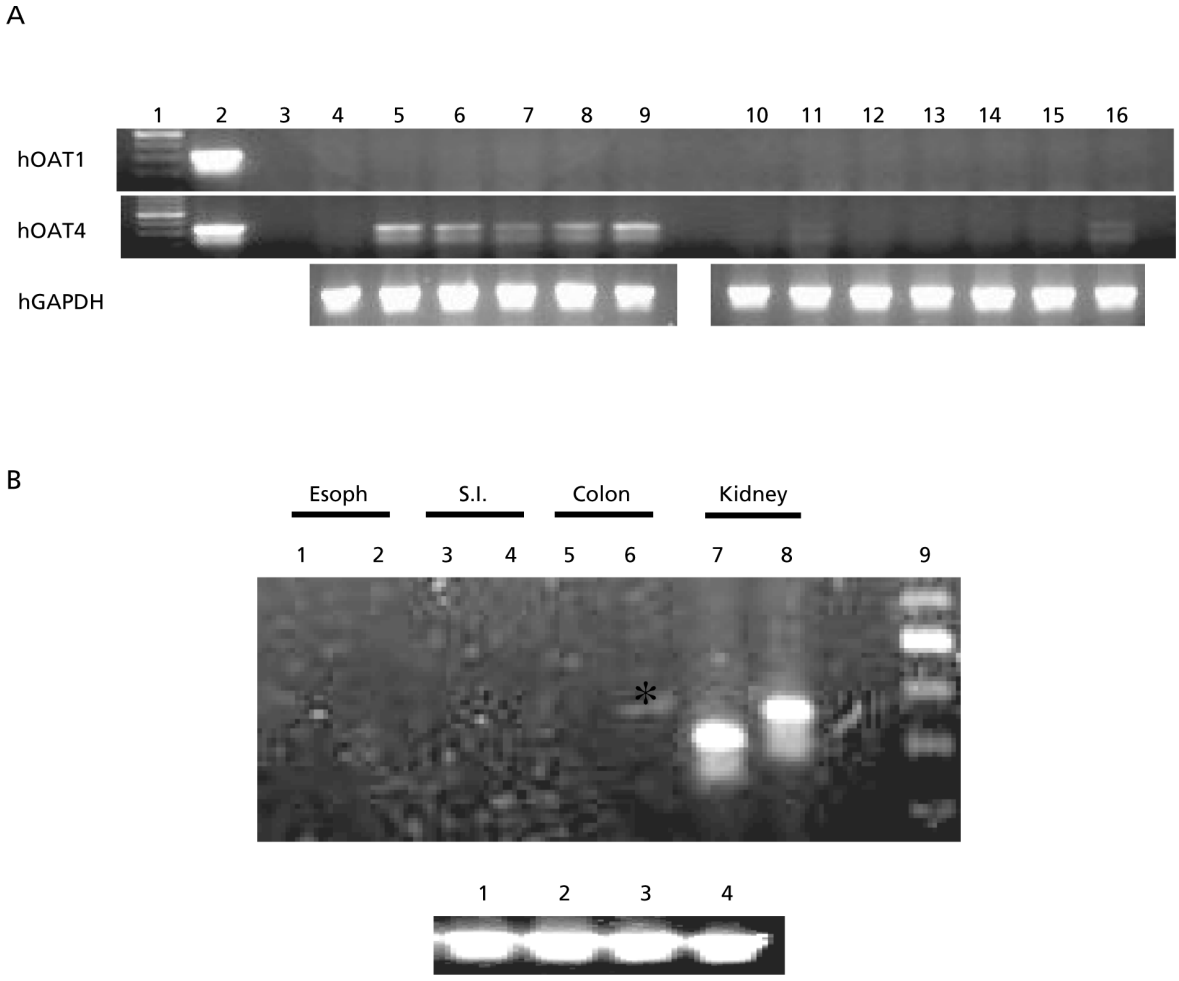

Figure 7 A. Expression of hOAT1 and hOAT4 in Caco-2 and HET-1A cells. Top panel – hOAT1, middle panel – hOAT4, bottom panel – hGAPDH (loading control). Lane 1 is 100 bp markers; lane 2 is positive plasmid controls; lane 3 is blank; lanes 4–9 are Caco-2 cells and lanes 10–16 are HET-1A cells. Lane 4 is 95% confluent; lanes 5, 6 and 10–12 are just confluent; lanes 7, 13 and 14 are 7 days post-confluent; lanes 8, 9, 15 and 16 are 14 days post-confluent. B. Expression of hOAT1 and hOAT4 in human oesophagus (Esoph), small intestine (S.I.), colon and kidney cDNA libraries. Top panel: lanes 1, 3, 5, 7 are hOAT1; lanes 2, 4, 6 and 8 are hOAT4. Lane 9 is 100 bp markers. The asterisk indicates hOAT4 expression in colon. Bottom panel – hGAPDH. Lanes 1–4 are oesophagus, small intestine, colon and kidney libraries, respectively.

involvement in the accumulation of EA in Caco-2 cells. A study also suggests it might be a pH-dependent mechanism (Hagenbuch & Meier 2003; Kobayashi et al 2003) with decreasing transport of substrate with increasing pH, the opposite of the effect observed for EA accumulation. The uptake of EA in the Caco-2 cells was thus examined in the presence of organic anion transport inhibitors to further implicate OATs. Accumulation was significantly inhibited by both DIDS and BSP (Saito et al 1996; Walters et al 2000), in particular by BSP in a dose-dependent manner, consistent with the involvement of potentially one or both families of transporters, although the sodium and pH effect already seems to exclude the OATPs.

In a parallel study using the *X. laevis* oocyte expression assay (Whitley et al 2005), transport of EA by hOAT1 and hOAT4 led us to examine OAT expression in the epithelial cells of the aerodigestive tract, which has not been thoroughly investigated. Mostly, the OATs have been localized via mRNA to the tubular cells of the kidney, the choroid plexus epithelium and the epithelial cells of the brain capillaries, as well as some other tissues such as placenta and liver (Kusuhara et al 1999; Cha et al 2000; Wright & Dantzler 2004). Our study is the first to show signs of expression of any OAT in Caco-2 cells, a model often used to make inferences about intestinal absorption of therapeutics. OAT4 is expressed on the apical membrane of renal proximal tubule cells (Wright & Dantzler 2004) and may also be expressed on the apical membrane of the Caco-2 cells, supporting the hypothesis that the high accumulation of EA in Caco-2 cells might be a result of expression of OAT4. The accumulation could also be a result of another OAT, such as an orthologue of the recently identified Oat6 (Monte et al 2004), or a currently unidentified OAT, which may be the case for HET-1A cells. As acceptable and available antibodies are developed, the role and expression of OAT4 and other OATs in Caco-2 cells will be tested.

Of further importance, based on the Caco-2 cell observations, OAT4 was also expressed in the large intestine of man, but not the small intestine. This correlates well with the origin of Caco-2 cells, being the human colon (Fogh et al 1977). Although it is known that as Caco-2 cells are cultured, they become more like small intestinal cells (Hidalgo et al 1989; Meunier et al 1995; Artursson & Borchardt 1997), their expression of OAT4, apparently a marker of the colonic type of cells, remains with these cells.

The mechanism of absorption of small organic anionic therapeutics across the intestine is not well understood. Some of these compounds are lipophilic enough in their non-ionized states to diffuse through the intestinal membranes to the portal circulation but for the majority of them the mechanism needs to be clarified. The expression of OATs or OAT-like transporters may play a role in the bioavailability of such therapeutics (Dresser et al 2002; Hagenbuch & Meier 2003; Jeong et al 2004). The transport properties of EA, a dietary compound, and many therapeutics could potentially lead to food–drug interactions in the intestine as well as in the kidneys (Whitley et al 2005).

In conclusion, we demonstrated that EA accumulates in epithelial cells along the aerodigestive tract in-vivo, confirming and greatly extending our previous findings in cultured Caco-2 cells (Whitley et al 2003). More in depth studies in Caco-2 cells showed that EA accumulation exhibits OAT-like properties. Finally, we showed for the first time OAT4 expression in Caco-2 cells as well as tentatively in the human large intestine. Further studies are necessary to make final conclusions regarding EA transporter(s) in the epithelial cells of the aerodigestive tract.

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