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Intestinal epithelial cell accumulation of the cancer preventive polyphenol ellagic acid—extensive binding to protein and DNA

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Abstract

Ellagic acid (EA), a polyphenol present in many berries, has been demonstrated to be preventive of esophageal cancer in animals both at the initiation and promotion stages. To be able to extrapolate these findings to humans we have studied the transcellular absorption and epithelial cell accumulation of [^{14}C]EA in the human intestinal Caco-2 cells. The apical (mucosal) to basolateral (serosal) transcellular transport of 10 μM [^{14}C]EA was minimal with a P_{app} of only 0.13×10^{-6} cm/s, which is less than for the paracellular transport marker mannitol. In spite of observations of basolateral to apical efflux, Caco-2 cell uptake studies showed high accumulation of EA in the cells (1054 ± 136 pmol/mg protein), indicating facile absorptive transport across the apical membrane. Surprisingly, as much as 93% of the cellular EA was irreversibly bound to macromolecules (982 ± 151 pmol/mg protein). To confirm the irreversible nature of the binding to protein, Caco-2 cells treated with 10 μM [^{14}C]EA were subjected to SDS–PAGE analysis. This resulted in radiolabeled protein bands trapped in the stacking gel, consistent with [^{14}C]EA-crosslinked proteins. Treatment of Caco-2 cells with 10 μM [^{14}C]EA also revealed irreversible binding of EA to cellular DNA as much as five times higher than for protein (5020 ± 773 pmol/mg DNA). Whereas the irreversible binding to protein required oxidation of EA by reactive oxygen species, this did not seem to be the case with the DNA binding. The avid irreversible binding to cellular DNA and protein may be the reason for its highly limited transcellular absorption. Thus, EA appears to accumulate selectively in the epithelial cells of the aerodigestive tract, where its cancer preventive actions may be displayed.

Keywords: Ellagic acid; Irreversible binding; Cellular uptake; Caco-2 cells; Transport

1. Introduction

Epidemiological evidence has long suggested that a diet rich in fruits and vegetables can reduce the risk of cancer [1]. Such diets are well known to contain a variety of chemicals that can affect the carcinogenic process in many ways [2].

Cancer of the esophagus is often present with advanced metastatic disease that makes the prognosis for this disease very poor [3–5]. Chemoprevention would seem like a useful approach for this difficult to treat cancer. The availability of a good animal model for esophageal tumors, including the esophageal-specific carcinogen *N*-nitrosomethylbenzylamine, has resulted in significant progress in this area. Certain berries, e.g. strawberries and raspberries, have been shown to be particularly effective against esophageal tumors using this rat model [6–8]. The mechanism of the chemopreventive action of these treatments is not clear but appears to involve multiple sites. One may be inhibition of carcinogen-bioactivation. Another could involve inhibition of binding of carcinogen to DNA. A third may be post-initiation effects involving inhibition of growth promotion.

One component in these berries thought to have a role in eliciting some of these effects is ellagic acid (EA), a symmetrical polyphenol (Fig. 1). In nature, EA exists in the free form and complexed in the form of ellagitannins, which are easily hydrolyzed [9–11]. Indeed, EA was effective against esophageal tumors in the same rat model [12,13]. Although EA was effective *in vivo* in the rat, the

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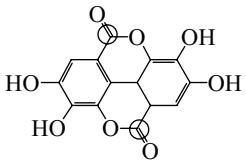

Fig. 1. Chemical structure of ellagic acid (EA). The sites of [^{14}C]-labeling are circled.

dose administered appeared higher than expected dietary intake. Animal studies suggest that only a limited fraction of EA is orally bioavailable [14]. There is no information on its disposition in humans. Thus, we do not know if EA will be able to be absorbed or if it will reach proposed sites of action, such as epithelial cells along the aerodigestive tract.

The present study reports on the transcellular absorption and cellular accumulation of EA, using the human intestinal cell line Caco-2 as a well accepted epithelial cell model that can be cultured as a monolayer on permeable membranes [15]. Access to radioactively labeled EA permitted dietary EA concentrations to be used in these experiments. The studies showed a complex behavior of EA with a high degree of irreversible binding to macromolecules.

2. Materials and methods

2.1. Materials

[^{14}C]EA (Fig. 1), was synthesized with a specific activity of 20 mCi/mmol at the Ohio State University Comprehensive Cancer Center. D-[1-^{14}C]-Mannitol (57.0 mCi/mmol) was purchased from Amersham Life Science, Inc. MK-571 was a gift from Dr. A.W. Ford-Hutchinson, Merck-Frosst Centre for Therapeutic Research. PSC 833 was a gift from Novartis Pharma AG. Pooled human liver microsomes were obtained from Gentest Corp., fetal bovine serum was purchased from Atlas and Hanks' Balanced Salt Solution (HBSS) and other cell culture medium components were obtained from Cellgro, Mediatech, Fisher Scientific. Other chemicals were purchased from Sigma–Aldrich or Fisher Scientific.

2.2. Cell culture

Human intestinal epithelial Caco-2 cells (ATCC) were cultured in Eagle's minimum essential medium with 10% fetal bovine serum, nonessential amino acids and penicillin/streptomycin in a 37° humidified incubator with 5% CO_2, as previously described [16]. Experiments were done in HBSS with 25 mM HEPES (pH 7.4) without phenol red. For pH effect on transport studies, the pH of HBSS was lowered to 5.5. For transport studies, the cells were seeded in 12 or 24 mm i.d. Transwell permeable polycarbonate inserts (0.4 μm pore size, Corning Costar Corp.) in 12- or 6-well plates at a density of 10^5 cells/cm^2. Cells were used for transport experiments at passage 35–75 at 19–28 days after seeding. Inserts were used for transport experiments when the transepithelial electrical resistance exceeded 275 and 200 Ω cm^2 in the 12 and 24 mm i.d. inserts, respectively.

2.3. Transport

Caco-2 cell layers, in 12-mm Transwell inserts, were washed twice for 30 min with warm HBSS. [^{14}C]EA was dissolved in DMSO and diluted to 10 μM with HBSS (final DMSO concentration 0.1%). This loading solution was added to either the apical or the basolateral chamber (0.5 or 1.5 mL, respectively). HBSS was added on the other side and the cell plates were returned to the incubator. In the experiments involving MK-571, a selective inhibitor of MRP2 [17], and PSC 833, a selective inhibitor of P-glycoprotein [18], the inhibitors were dissolved in HBSS and added to both sides in the second 30-min wash and with EA during the incubation. All solutions were made fresh immediately prior to each experiment. Samples were taken from the apical and basolateral chambers after various incubation times and subjected to liquid scintillation spectrometry. [^{14}C]-Mannitol (0.1 μCi) was added to the apical chamber in parallel Transwell inserts and the transport into the basolateral chamber at 60 min was analyzed by liquid scintillation spectrometry.

2.4. Cellular uptake of ellagic acid

In experiments designed to compare transport with cellular uptake of EA, 10 μM [^{14}C]EA in HBSS (1.5 mL) was added to the apical chamber of 24 mm i.d. Transwells seeded with Caco-2 cells. Following 60 min incubation, the basolateral chamber buffer (2.5 mL) was analyzed for [^{14}C]EA. The inserts were washed with 3 volumes of ice-cold buffer. The Transwell membranes with cells were excised and the cells were digested with 0.5 M sodium hydroxide overnight and analyzed by liquid scintillation spectrometry.

In experiments designed to compare extractable vs. non-extractable cellular uptake of EA, Caco-2 cells grown in 6-well plates were incubated with 10 μM [^{14}C]EA in HBSS for 60 min. The cell monolayers were rinsed three times with ice-cold buffer and scraped from the wells into 2 mL of methanol. Following shaking for 30 min, the lysed cell suspensions were pelleted. The pellets were digested overnight in 0.5 M sodium hydroxide and the cell digests and the methanol extracts were analyzed by liquid scintillation spectrometry. Protein content was determined using the Coomassie® Plus Protein Assay Reagent kit from Pierce.

2.5. Ellagic acid binding to protein

Confluent Caco-2 cells in 6-well plates were incubated with 1.5 mL 10 μM [^{14}C]EA in HBSS for 0, 15, and

60 min. The cell monolayers were then rinsed three times with ice-cold buffer. The cells were scraped into buffer and homogenized on ice with a Polytron homogenizer (2 × 15 s). An aliquot of the homogenate was analyzed by liquid scintillation spectrometry before addition of 25% trichloroacetic acid to precipitate the proteins. After centrifugation, the pellet was sequentially extracted with 5% trichloroacetic acid and twice each with 80% methanol, hot 80% methanol, methanol/diethyl ether (50/50, v/v), and 80% methanol [19] and finally digested with 0.5 M sodium hydroxide. The radioactivity in the pellet after this exhaustive extraction procedure represents irreversible binding of [^{14}C]EA to cellular protein. The protein content was determined as above.

Pooled human liver microsomes (200 μg) were incubated with [^{14}C]EA (25 μM) in 100 mM sodium phosphate buffer (pH 7.0) for 30 min. Other samples contained boiled microsomes or NADPH (2 mM). Still other samples contained horseradish peroxidase (80 U) and hydrogen peroxide (110 μM) in the absence or presence of glutathione (5 mM). After incubation, microsomal protein was extracted similarly as Caco-2 cell protein.

2.6. Gel electrophoresis

Confluent monolayers of Caco-2 cells, in 6-well plates, were treated with 25 μM [^{14}C]EA for 60 and 120 min. The cell monolayers were washed and lysed in 500 μL Laemmli sample buffer with β-mercaptoethanol [20]. The cell lysates (900 μg protein) were loaded on 8–20% gradient SDS–polyacrylamide gels. After electrophoresis, the gels were stained with Coomassie blue, dried on filter paper and subjected to autoradiography. The films were developed after one month at −70°.

2.7. Ellagic acid binding to DNA

Confluent Caco-2 cells in 6-well plates were incubated with 10 μM [^{14}C]EA in HBSS. The cell monolayers were then rinsed three times with ice-cold buffer before the addition of lift buffer (1 mM EDTA in Tris-buffered saline, pH 8) and incubation at 37° until detached. The cells from two wells were combined, pelleted and resuspended in swell buffer (with spermidine, spermine, EDTA, EGTA) and Triton X-100. After 10 min on ice, the lysed cells were spun down. The pellets were resuspended in swell buffer and the nuclei centrifuged through a 30% sucrose cushion. The nuclear pellets were resuspended in Tris/EDTA buffer with RNAse, proteinase K and SDS to digest RNA and protein overnight [21]. The samples were extracted four times with phenol/chloroform/isoamyl alcohol (Amresco). To the final aqueous phase was added sodium acetate to 1 M and ice-cold ethanol to precipitate the DNA. The DNA pellets were washed twice with cold ethanol and dissolved in water. The purity of the DNA was evaluated as the 260/280 nm UV absorbance ratio. In all cases this ratio was 1.6–0.8. The amount of [^{14}C]EA bound to DNA was obtained by liquid scintillation spectrometry and the amount of DNA by its 260 nm absorbance.

In vitro binding of EA was performed with calf thymus DNA (1.6 mg/mL sodium phosphate buffer, pH 7.0), adapted from [21]. [^{14}C]EA (25 μM) was incubated for 5 min in either buffer alone or with horseradish peroxidase (80 U) and hydrogen peroxide (110 μM) with or without glutathione (5 mM) followed by incubation with DNA for 30 min. The incubates were extracted twice with water-saturated *n*-butanol and twice with phenol/chloroform/isoamyl alcohol. DNA purification and quantitation was performed similarly as Caco-2 cell DNA.

2.8. Distribution ratio of EA

10 μM [^{14}C]EA in 100 mM sodium phosphate buffer, pH 7.0, was shaken with an equal volume of buffer-saturated 1-octanol. After separation of the phases, aliquots of buffer and 1-octanol were analyzed by liquid scintillation spectrometry.

2.9. Calculations and statistics

The apparent permeability coefficients (P_{app}), expressed in cm/s [22], were calculated as $\Delta Q/\Delta t \times 1/60 \times 1/A \times 1/C_0$, where $\Delta Q/\Delta t$ is the permeability rate (μg/min), A is the surface area of the membrane (cm^2), and C_0 is the initial concentration in the donor chamber (μg/mL).

The statistical significance of differences between different incubation conditions was evaluated using Student's two-tailed unpaired *t*-test.

3. Results

The concentration of EA employed in this study was based on the content of EA in one cup of fresh raspberries, i.e. about 30 mg [7]. Assuming a gastric fluid volume of 500 mL, the resulting concentration would be in the range of 200 μM [16]. Because of the limited solubility of EA, i.e. 30 μM, we elected to use a concentration of 10 μM, i.e. considerably less than maximum dietary intake.

3.1. Transcellular transport

The transcellular transport of 10 μM [^{14}C]EA across Caco-2 cells grown in Transwells after loading EA on either the apical or basolateral side, is shown in Fig. 2A. Transport occurred in both directions but was much more pronounced in the basolateral to apical direction, i.e. efflux. Both apical to basolateral and basolateral to apical transport was linear for up to 60 min, then appeared to become saturable, maybe due to metabolism of EA. Repeated measures at 60 min and calculation of P_{app} values gave the data in Fig. 2B, demonstrating that the

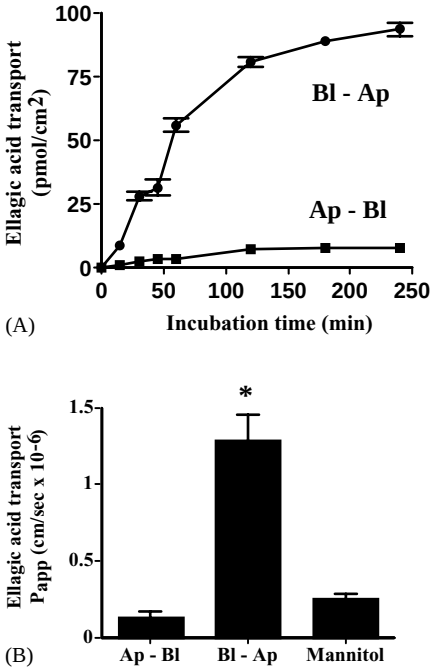

Fig. 2. Transport of EA (10 μM) across the Caco-2 cell monolayer. (A) Time-course of apical to basolateral (Ap-Bl) and basolateral to apical (Bl-Ap) transport. Data are mean ± SEM. Bl-Ap is higher than Ap-Bl at all time points ($P < 0.0001$; N = 5–11). (B) Transport rates expressed as P_{app} for EA Ap-Bl and Bl-Ap and for mannitol Ap-Bl at 60 min. Data are mean ± SEM (N = 15 for EA; N = 6 for mannitol). *Higher than Ap-Bl; $P < 0.0001$.

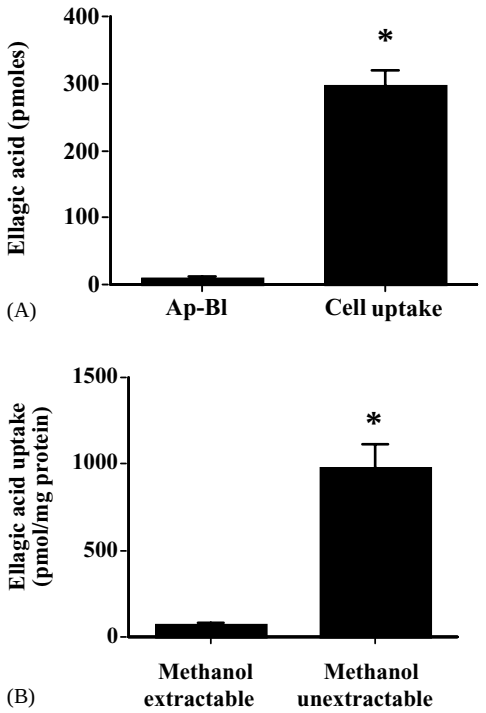

Fig. 3. Caco-2 cell accumulation of EA (10 μM). (A) Sixty minutes cell uptake vs. apical to basolateral (Ap-Bl) transport in Transwells. Data are mean ± SEM (N = 6 for Ap-Bl; N = 12 for cell uptake). *Higher than Ap-Bl; $P < 0.0001$. (B) Sixty minutes cell uptake, methanol-extractable vs. methanol-unextractable, in 6-well plates. Data are mean ± SEM (N = 18). *Higher than methanol extractable; $P < 0.0001$.

P_{app} for apical to basolateral transport of $0.13 \pm 0.04 \times 10^{-6}$ cm/s (mean ± SEM; N = 15) was lower than for the paracellular transport marker mannitol of $0.26 \pm 0.03 \times 10^{-6}$ cm/s (N = 6), although not statistically significantly different. On the other hand, the basolateral to apical efflux of $1.29 \pm 0.16 \times 10^{-6}$ cm/s (N = 15) exceeded that of the apical to basolateral absorption by as much as 10-fold ($P < 0.0001$).

In attempts to identify the potential efflux transporter involved, 60-min transport experiments with [^{14}C]EA were carried out in the presence of either 30 μM MK-571, an inhibitor of apical MRP2 [17], or 0.1 μM PSC-833, an inhibitor of apical P-glycoprotein [18]. Neither MK-571 nor PSC-833 affected the transport of EA (data not shown) at these concentrations, well known to inhibit MRP2 and P-glycoprotein. When both transport inhibitors were used simultaneously, there was about 35% inhibition of transport, however, not significantly different from control. Similar experiments in the MRP2-transfected MDCK cell line [23] further confirmed that MRP2 does not play a role in EA transport (data not shown). As EA has acidic properties with one pK_a value of around 6.3 [24], we also examined if lowering the pH of the transport buffer from 7.4 to 5.5 had an effect on transport, but it did not.

3.2. Cellular accumulation

Even though transcellular apical to basolateral absorption was very low, it could not be excluded that EA entered the cell via the apical membrane and accumulated in the cell. This was tested by incubating the Caco-2 cells in 24-mm Transwells with 10 μM [^{14}C]EA in the apical chamber. After 60 min, the basolateral compartment buffer was taken for analysis of apical to basolateral transport as above. Consistent with the results above, only very small amounts of EA, i.e. 9.8 ± 1.8 pmol were recovered (Fig. 3A). When the Transwell membrane with the cells was washed, excised from the Transwell, and the cell pellet was digested with sodium hydroxide, surprisingly large amounts of radioactivity, 299 ± 22 pmol EA, were recovered, indicating a high accumulation of EA in the Caco-2 cells (Fig. 3A). The calculated concentration of EA in the Caco-2 cells was 728 ± 71 pmol/mg protein.

Identical incubations were done in Caco-2 cells, grown in 6-well plates, thus with only the apical membrane of these polarized cells exposed to EA. After washing the cell surface with cold buffer as above, the cells were scraped off into methanol and transferred to Eppendorf tubes. After vigorous shaking and centrifugation, the supernatant and the cell pellet were analyzed by liquid scintillation spectrometry (Fig. 3B). Very little of the cellular [^{14}C]EA was extractable, suggesting covalent binding of EA to protein. Most importantly, the total cell content of EA in this experiment, i.e. 1054 ± 136 pmol/mg protein, was similar to that in Fig. 3A.

To further examine the high cell uptake as well as the covalent binding postulate, Caco-2 cells, grown in 6-well

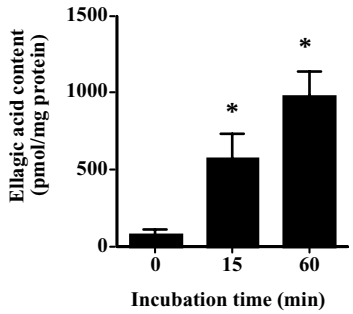

Fig. 4. Irreversible binding of EA (10 μM) to Caco-2 cell protein. Caco-2 cells cultured in 6-well plates were incubated with EA for 0–60 min. Cell protein was precipitated and, following exhaustive solvent extraction, EA content was determined. Data are mean ± SEM (N = 15–22). *Higher than at 0 min; $P < 0.01$.

plates, were again incubated with 10 μM [^{14}C]EA. After washing the cells as above, the cells were scraped off into aqueous buffer and homogenized. Total protein was precipitated with trichloroacetic acid and extensively extracted with organic solvents to remove any noncovalently bound [^{14}C]EA using the exhaustive protein extraction technique [19]. The results in Fig. 4 demonstrate a time-dependent increase in the irreversible binding of [^{14}C]EA to protein. It should be noted that because of the experimental setup 0 min is probably closer to about 2 min. The irreversibly bound cellular content of EA at 60 min, 982 ± 151 pmol/mg protein, was similar to the total cellular content in Fig. 3B.

To confirm the covalent protein binding of EA, and start to explore to which cellular proteins EA may covalently bind, total Caco-2 cell lysates, after incubation with [^{14}C]EA, were subjected to SDS–PAGE, under reducing conditions, followed by autoradiography (Fig. 5). As expected, Coomassie blue staining showed a large number of protein bands over the 10–220 kDa molecular weight range of the gel. In the corresponding autoradiogram, there were no clearly defined radioactive bands. All of the radioactivity associated with [^{14}C]EA binding to proteins was found in the stacking gel, consistent with crosslinked proteins. Similar results were observed under nonreducing conditions (data not shown). It should be pointed out that, because of the low specific activity of the [^{14}C]EA used, the autoradiograms took a month to develop.

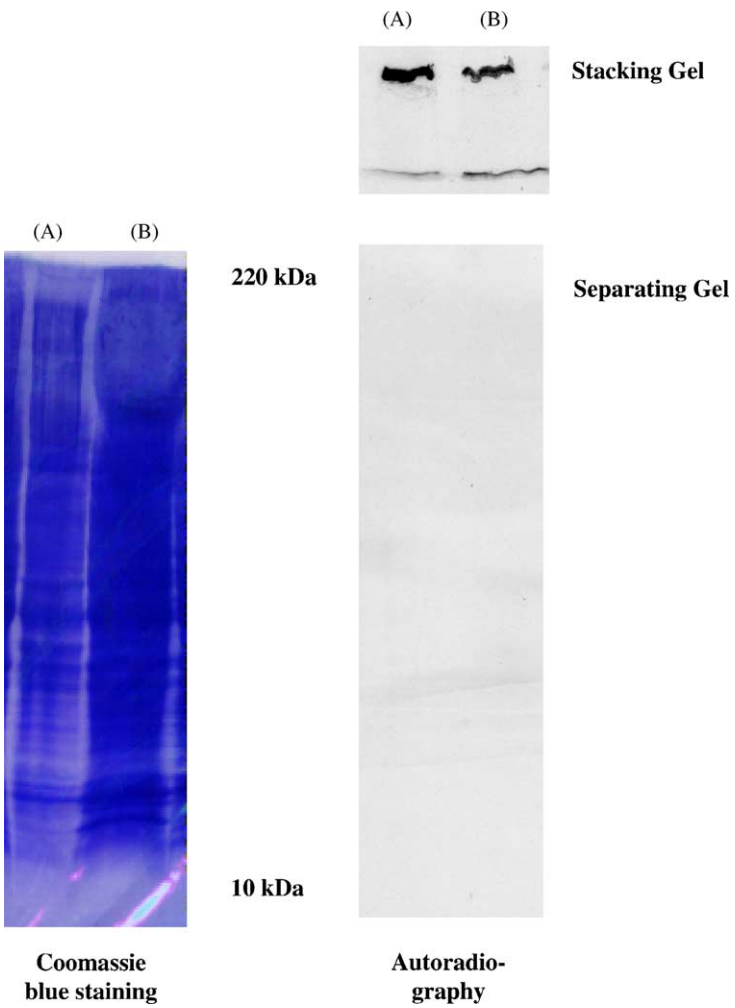

Fig. 5. SDS–PAGE and autoradiography of Caco-2 cell lysate after (A) 120 min; (B) 60 min incubations of Caco-2 cells with 10 μM [^{14}C]EA. Each lane contained approximately 300 μg cellular protein.

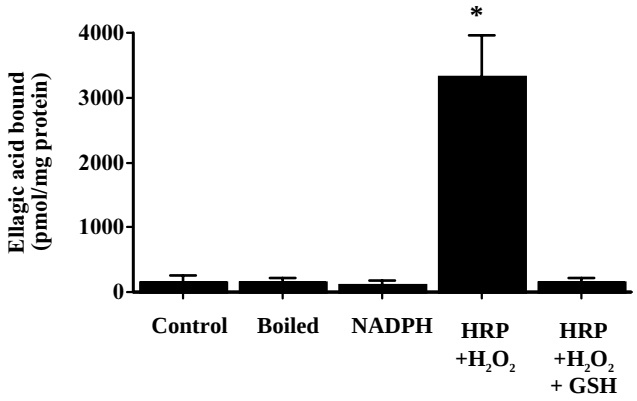

Fig. 6. Irreversible binding of EA (25 μM) to human liver microsomal protein (HLM). HLMs (200 μg) were incubated with EA for 30 min (control). Other samples contained boiled microsomes or NADPH (2 mM). Still other samples contained horseradish peroxidase (80 U) and hydrogen peroxide (110 μM) in the absence or presence of glutathione (5 mM). After exhaustive extraction of protein, [^{14}C]EA content was determined. Data are mean ± SEM (N = 3). *Higher than all; $P < 0.01$.

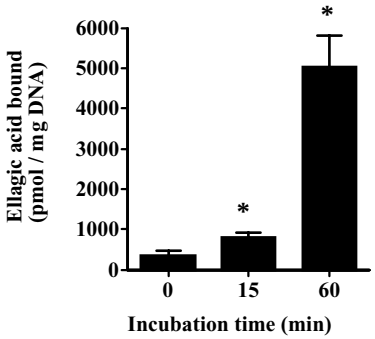

Fig. 7. Irreversible binding of EA (10 μM) to Caco-2 cell DNA. Caco-2 cells cultured in 6-well plates were incubated with EA for 0–60 min. After DNA extraction and purification, [^{14}C]EA content was determined. Data are mean ± SEM (N = 9). *Higher than at 0 min; $P < 0.05$.

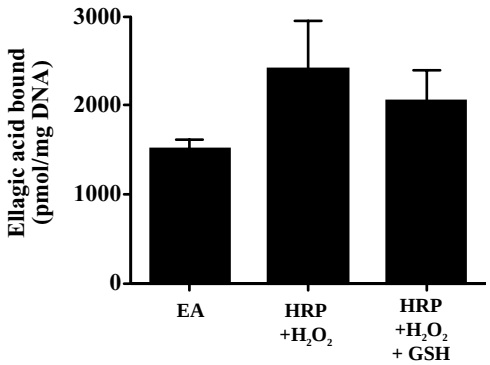

Fig. 8. Irreversible binding of EA (25 μM) to calf thymus DNA. EA was incubated with either buffer alone or hydrogen peroxide (110 μM) and horseradish peroxidase (80 U) in the presence or absence of glutathione (5 mM) for 5 min followed by addition of calf thymus DNA for 30 min. After DNA extraction and purification, [^{14}C]EA content was determined. Data are mean ± SEM (N = 9). There were no significant differences between treatments.

To determine whether [^{14}C]EA could bind spontaneously to cellular protein or required prior oxidation in the cell, we incubated [^{14}C]EA with human liver microsomal protein as an acceptor. In the presence of NADPH, i.e. a cytochrome P450 competent preparation, there was a slight decrease in covalent binding compared to control or boiled microsomes (Fig. 6). However, in the presence of hydrogen peroxide and horseradish peroxidase there was a dramatic increase in the covalent binding. Additional HPLC experiments demonstrated that these as well as lower concentrations of hydrogen peroxide and horseradish peroxidase, compatible with physiologic concentrations, completely oxidized [^{14}C]EA to polar, unknown products (data not shown). When physiologic concentrations of glutathione (5 mM) were added, this covalent binding was completely abolished (Fig. 6).

In further experiments, we incubated Caco-2 cells, cultured in 6-well plates, with 10 μM [^{14}C]EA, as above. After washing the cells, the cells were detached and lysed. The nuclear pellet, protein, and RNA were digested overnight. The cellular DNA was purified by phenol/chloroform/isoamyl alcohol extraction, washed with ethanol and dissolved in water [21]. The solution was analyzed for DNA purity (OD 260/280 = 1.6–0.8), concentration, and [^{14}C]EA content. As seen in Fig. 7, there was a time-dependent increase in the irreversible binding of [^{14}C]EA to DNA. The binding of EA to DNA at 60 min was about five times higher than the irreversible binding to protein on a per mg basis (cf. Fig. 4).

As with the irreversible binding of [^{14}C]EA to microsomal protein above we tested whether oxidation was a necessary prerequisite for binding to DNA, using calf thymus DNA as the acceptor. Similar to the cell experiment in Fig. 7 we observed a high level of irreversible binding (Fig. 8). In the presence of hydrogen peroxide and horseradish peroxidase, in contrast to the microsomal protein binding in Fig. 6, there was only a slight, not significant, increase in the binding to DNA. When glutathione was present, the binding was unaffected.

4. Discussion

Ellagic acid, EA, has been shown to have the potential to be a useful cancer preventive and/or chemotherapeutic agent [13], however, too little is known about its ability to be absorbed and distributed in the human body. In the present study we used the human intestinal epithelial Caco-2 cell to study both transport and cellular uptake of EA using [^{14}C]-labeled polyphenol.

EA showed very limited apical to basolateral absorption with a P_{app} value of 0.13×10^{-6} cm/s, which is only about one-half of the paracellular transport marker mannitol. In part, this low transcellular absorption could be attributed to basolateral to apical efflux, which was about 10 times higher than the absorption. The mechanism of this efflux could not successfully be resolved. Neither MRP2 nor

P-glycoprotein, two well-known apical efflux transporters [25,26], or a proton-dependent transporter, appeared to be involved.

Whereas the transcellular absorption of EA was very low, surprisingly, the cellular uptake was extensive. This implied that EA freely could cross the apical membrane but that further transport across the monolayer was highly restricted. Interestingly, almost all of the EA being taken up by the Caco-2 cells appeared to be irreversibly bound to macromolecules. The binding to DNA was about five times higher than to protein, per mg DNA or protein.

Previous studies have demonstrated EA to either react with carcinogen [27] or inhibit carcinogen binding to DNA [28,29]. Also, irreversible binding to DNA has been reported in organ explants of rats [30,31]. From these studies the binding appeared to be spontaneous, thus, not involving enzymatic activation. The present study is the first to report a very rapid and extensive irreversible binding of EA to DNA in a human epithelial cell during the course of its enteric absorption. In a previous study we demonstrated irreversible binding of the flavonoid [^{14}C]quercetin also to DNA in Caco-2 cells [15]. The binding of EA to DNA was as much as 500 times higher than for quercetin. This large difference may be due to the greater ability of EA than quercetin to intercalate with the double-stranded DNA molecule [32]. For quercetin the binding appeared to be catalyzed by reactive oxygen species (ROS) [15], which converted this polyphenol to quinoid intermediates. The same did not appear to be the case with EA. Thus, the oxidation of [^{14}C]EA by hydrogen peroxide and horseradish peroxidase did not result in a significant increase in the DNA binding. Reversed-phase HPLC clearly showed that these conditions led to complete oxidation of [^{14}C]EA to polar products. Thus, the irreversible binding of [^{14}C]EA to DNA does not seem to require prior oxidation but may be, as previously suggested, a high affinity intercalation with the DNA [28,30,32,33], an interaction that is not dissociated by the classic phenol/chloroform extraction assay.

No previous studies have reported on irreversible binding of EA to protein. Also this binding was higher for EA than for quercetin [15], about 10-fold. In contrast to the binding to DNA the binding to microsomal protein, used as the acceptor, required oxidation by hydrogen peroxide and horseradish peroxidase, resulting in a dramatic increase in the binding. Most importantly, this binding was completely abolished by glutathione. The covalent binding of quercetin to cellular protein gave rise to multiple distinct radiolabeled protein bands by SDS–PAGE and autoradiography [15]. In contrast, the covalent binding of EA to protein did not give rise to any labeled protein bands in the separating gel. Instead, all of the radioactivity was confined to proteins in the stacking gel. This may likely have been due to large molecular weight adducts potentially formed by EA acting as a macromolecular cross-linker. EA is a symmetrical molecule (Fig. 1) with potential to be oxidized to a di-radical or di-quinone with high reactivity towards sulfhydryl and amino groups of proteins [34].

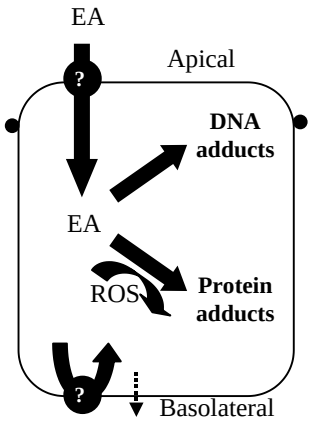

Fig. 9. Proposed schematic model of EA disposition in epithelial Caco-2 cells. ROS, reactive oxygen species; ? denotes potential transporters.

A further site of macromolecular binding of EA, that should be addressed in future studies, is binding to proteins or lipids in the cell membrane, thus, not requiring transmembrane transport. Considering the hydrophilicity of EA, interaction with lipid membranes may, however, be limited.

The observations in this study taken together lead to a proposed scheme involved in EA uptake and disposition by the epithelial Caco-2 cells (Fig. 9), which may be shared by other types of epithelial cells of the aerodigestive tract, e.g. esophageal cells in which EA is protective against tumor formation [6–8]. Thus, after entering the cell via the apical membrane, possibly by passive diffusion, EA is intercalated and/or bound to DNA. Also, a large fraction of EA is oxidized, presumably by ROS such as hydrogen peroxide and/or hydroxyl radicals to presently unknown reactive quinones which bind at high levels to protein. Both of these macromolecular binding processes are the likely reason for the very limited transcellular absorption of EA towards the basolateral side.

However, much less obvious membrane transport may shed further light on the extensive epithelial cell accumulation of EA. Considering the low octanol/pH 7.0 buffer distribution ratio for EA of 0.27 ± 0.01, it does not seem likely that EA will cross the apical membrane by passive diffusion alone. Thus, a currently unknown absorptive transporter could be an important contributor (Fig. 9). In addition, basolateral absorption may keep EA in the cell (Fig. 9). Such a transporter may be one similar to OATP2, shown to be critically important for hepatocyte extraction and elimination of bilirubin from the blood [35]. Thus, further work will be needed to support the scheme in Fig. 9.

The value of the Caco-2 cell culture model in this study is 2-fold. As shown in numerous previous studies, summarized in [15], the Caco-2 cells have a high predictive value regarding human intestinal absorption. Thus, based

on our observations, EA will likely not be absorbed into the circulation but rather accumulate in the epithelial cells along the aerodigestive tract. On the other hand, being a cancer cell, the Caco-2 cell may behave as other cancer cells regarding oxidation of EA. Thus, many cancer cells have higher levels of ROS, or lower levels of ROS-inactivating enzymes [36,37] than normal cells.

In summary, this study demonstrates highly effective accumulation of EA by epithelial cells of the intestine that may also be important for other epithelial cells in the aerodigestive tract. This view is supported by previous *in vivo* studies demonstrating very limited accumulation of EA at other tissue sites following oral administration of EA [14,38]. Highly specific membrane transporters may further help understand this accumulation. The finding that EA after entering the cells becomes tightly bound to DNA is consistent with previous observations and remains an intriguing observation regarding the cancer preventive effects of EA. The finding of high covalent binding to cellular protein is a novel finding for EA, suggesting an important contribution of ROS in the cellular disposition of EA. This will require further studies to be able to assess its importance in cellular protection vs. possible toxicity.

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