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Dissimilar *In Vitro* and *In Vivo* Effects of Ellagic Acid and Its Microbiota-Derived Metabolites, Urolithins, on the Cytochrome P450 1A1

Antonio González-Sarrías, María Azorín-Ortuño, María-Josefa Yáñez-Gascón, Francisco A. Tomás-Barberán, María-Teresa García-Conesa,* and Juan-Carlos Espín

Research Group on Quality, Safety, and Bioactivity of Plant Foods, Department of Food Science and Technology, Centro de Edafología y Biología Aplicada del Segura−Consejo Superior de Investigaciones Científicas (CEBAS−CSIC), Post Office Box 164, 30100 Campus de Espinardo, Murcia, Spain

This research shows that the dietary polyphenol ellagic acid (EA) and its colonic metabolites, urolithin-A (3,8-dihydroxy-6*H*-dibenzo[*b*,*d*]pyran-6-one, Uro-A) and urolithin-B (3-hydroxy-6*H*-dibenzo[*b*,*d*]pyran-6-one, Uro-B), modulate phase I and phase II detoxifying enzymes in colon cancer Caco-2 cells. EA and the urolithins, at micromolar concentrations achievable in the colon from the diet, induced the expression and activity of CYP1A1 and UGT1A10 and inhibited several sulfotransferases. As a result, the synthesis of glucuronides was favored over sulfated conjugates in the treated cells. *In situ* exposure to these compounds dissolved in buffer also led to the induction of CYP1A1 in the rat colon. However, *in situ* exposure to the compounds dissolved in oil or oral supplementation of the single compounds or pomegranate extract (PE) added to the feed failed to induce CYP1A1 in the colon mucosa. These results suggest that EA and urolithins may exert some blocking chemopreventive effects in the colon but that this effect is critically affected by interfering factors, such as the food matrix nature.

KEYWORDS: Pomegranate; CYP1A1; cancer; glucuronosyltransferases; sulfotransferases; Caco-2; colon mucosa; xenobiotic metabolism

INTRODUCTION

Prevention of carcinogenesis may occur by several mechanisms including the blocking of tumor initiation and/or the suppression of tumor progression. One way of blocking DNA damage and reducing cell malignization is by modulating phase I and phase II enzymes to enhance the detoxification of carcinogenic compounds (*1*). Many procarcinogens are activated by phase I cytochrome P450 monooxygenase enzymes (CYPs) usually yielding more reactive species. These reactive molecules may become inactivated by phase II enzymes [UDP-glucuronyl transferases (UGTs), sulfotransferases (SULTs), etc.] to more soluble compounds ready for excretion (*2*). Phase I and phase II enzymes form large families of isoenzymes expressed in many tissues, including the colon mucosa (*3*, *4*). Within the CYP1 family, CYP1A1 and CYP1B1 are responsible for the metabolism of dietary carcinogens, such as the polycyclic aromatic hydrocarbons (PAHs) associated with colon cancer development (*2*). Increased CYP1A1 and CYP1B1 expression and activity have been linked to a high risk of colorectal cancer (*5*, *6*). The regulation of CYPs has the potential to protect against exposure to carcinogens and to prevent cancer development. Commonly, induction of CYPs has been considered detrimental because of the likely formation of reactive damaging metabolites, whereas inhibition of the CYP activity may reduce the activation of procarcinogens. However, the induction of CYPs may also decrease the carcinogenicity of some chemicals (*7*). Detoxification by inducible CYP1A1 has been reported to be more important than activation upon oral exposure to benzo[*a*]pyrene in mice (*8*). Induction of phase II enzymes also represents a mechanism of anticarcinogenesis. The final outcome will depend upon the type of chemical and the coordinated regulation of different enzymes and pathways (*1*).

Flavonoids have been shown to modulate phase I or phase II enzymes at the mRNA, protein, and/or activity level both *in vitro* and *in vivo*, but the effects depend upon their structure, concentration, and experimental model (*9*, *10*). For example, an apple juice extract and some of its flavonoid constituents weakly induced CYP1A1 mRNA and protein but inhibited the enzyme activity and suppressed the induction of CYP1A1 by tetrachlorodibenzo-*p*-dioxin in human colon cancer Caco-2 cells (*11*). Another flavonoid-rich fruit juice, pomegranate juice, also inhibits human liver CYP3A and CYP2C9 *in vitro* activities (*12*, *13*). The consumption of pomegranate juice decreases total hepatic CYP content as well as the expression of CYP1A2 and CYP3A in mice (*14*). These results suggest that some component (s) in the pomegranate may inhibit certain CYPs with putative

*To whom correspondence should be addressed. Telephone: +34-968-39-6276. Fax: +34-968-39-6213. E-mail: mtconesa@cebas.csic.es.

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chemopreventive effects. Pomegranate is rich in the polyphenols ellagitannins (ETs), which are hydrolyzed in the gut to release ellagic acid (EA), which is further metabolized by the intestinal flora to yield the urolithins (*15*). EA or the urolithins are poorly absorbed, whereas these compounds can be found at relatively high concentrations in the colon, in particular, the metabolite urolithin-A (3,8-dihydroxy-6*H*-dibenzo[*b*,*d*]pyran-6-one, Uro-A). Small quantities of urolithin-B (3-hydroxy-6*H*-dibenzo-[*b*,*d*]pyran-6-one, Uro-B), urolithin-D (*tetra*-hydroxy-dibenzopyranone, Uro-D), or urolithin-C (trihydroxy-dibenzopyranone, Uro-C) can also be detected (*16*). ETs, EA, and the urolithins exhibit anticancer activities against cancer colon cells and animal models (*17*−*19*), but the molecular mechanisms involved are only beginning to emerge (*20*). EA has been shown to inhibit CYP1A1 activity in the rat liver (*15*). However, it is not known whether EA and its colonic derivatives, the urolithins, may exert their chemopreventive effects in the colon by affecting CYPs and phase II enzymes.

The aim of this study was to investigate whether EA and/or its metabolites, Uro-A and Uro-B, can modulate phase I and/or phase II enzymes in the colon, which may be indicative of some tumor initiation blocking mechanisms of action. For this purpose, we examined whether these compounds alter the expression and activity of phase I and phase II enzymes in an *in vitro* model of human colon cancer, Caco-2 cells, and in the rat colon mucosa. In addition, we investigated whether EA and the urolithins are further metabolized by these enzymes to any form of soluble conjugates.

MATERIALS AND METHODS

Materials. EA (95% purity) was purchased from Sigma-Aldrich (Steinheim, Germany) and purified to almost 100% purity as described elsewhere (*20*). Uro-A (95% purity) and Uro-B (98% purity) were chemically synthesized by Kylolab S.A. (Murcia, Spain). Pure punicalagin standard was obtained from pomegranate peel according to the protocol of Cerdá et al. (*21*). Pomegranate extract ("Nutragranate") (PE) was kindly provided by Nutracitrus S.L. (Elche, Spain). Quercetin was from Sigma (St. Louis, MO).

Cell Culture. The human colon cancer cell line Caco-2 was obtained from the American Type Culture Collection (ATCC) (Rockville, MD), and the cells grown as previously reported (*20*). Cells were seeded at 10^4 cells cm^{-2} on 6-well plates (Nunc, Roskilde, Denmark), allowed to adhere for 48 h, and treated on day 3 after seeding as follows: (i) 10 μM EA, (ii) 40 μM Uro-A, (iii) 40 μM Uro-B, (iv) a mixture of 40 μM Uro-A, 40 μM Uro-B, and 10 μM EA (MIX). All of the compounds were dissolved in dimethylsulfoxide (DMSO) (< 0.5% in the culture medium) and filtered (0.2 μm) prior to addition to the culture media. Control cells were treated with DMSO. After the treatments, control and treated Caco-2 cells were directly lysed in the plates for RNA extraction or used to determine CYP1A1 and phase II enzyme activity as described in the corresponding sections. Experiments were all performed in triplicate.

Cellular Uptake. To examine the ability of Caco-2 cells to form and excrete derived metabolites, cells (10^6) were incubated for 8, 24, and 48 h with 40 μM Uro-A, 40 μM Uro-B, or the MIX in culture media without phenol red according to a published protocol (*22*). At the end of each treatment, samples of media and cell lysates (100 μL) were analyzed by liquid chromatography−tandem mass spectrometry (LC−MS/MS).

Animals and Experimental Design. Sprague−Dawley rats (200−250 g) were provided by the Experimental Animal Facility of the University of Murcia (Spain). All animal studies followed a protocol approved by the local animal ethics committee and the local government and were in accordance with the recommendations of the European Union regarding animal experimentation (Directive of the European Council 86/609/EC). Rats were housed 3−4 to a cage in a room with controlled temperature (22 ± 2 °C), 55 ± 10% relative humidity, and a 12 h light−dark cycle. They were fed with a rat standard chow (Panlab, Barcelona, Spain) containing 14.5% proteins, 63.9% carbohydrates, and 4% fat (3.2 kcal/g) until the start of the experiments. Diet and tap water were administered *ad libitum*. Animals were randomly assigned to two experimental protocols.

In situ model: rats (n = 7 animals per treatment) fasted for 12 h were anaesthetized by intraperitoneal administration of a mixture (1:1, v/v; 1 mL/kg body weight) of xylazine (Xilagesic 2%) (Calier Laboratories, Barcelona, Spain) and ketamine (Imalgene 1000) (Merial Laboratories, Barcelona, Spain). A longitudinal midline incision was made in the abdomen, and the intestines were carefully manipulated to avoid blood supply disturbances. The colon was then exposed; incisions were made at both ends of it; and the isolated segment was rinsed with phosphate-buffered saline (PBS) until the effluent solution was clear. The distal orifice was ligated, and the incubation testing solution was slowly infused into the colon with a syringe followed by ligation of the proximal orifice. The colon and the intestines were placed back into the abdomen and covered with a surgical dressing. To maintain normal body temperature, the rats were kept under a heating lamp during the 2 h incubation period. The test compounds (EA and/or urolithins) were incubated and dissolved in either PBS or sunflower oil at the same final concentrations described for the cell culture experiments. Controls were carried out with PBS or sunflower oil alone. At the end of the experiment, the rats were killed by decapitation, the colon was quickly removed, rinsed with PBS, and opened longitudinally, and the mucosa was scrapped off with a glass slide. Mucosal scrapings were prepared for RNA extraction (four animals per treatment) by direct suspension in lysis buffer or for total protein extraction (three animals per treatment) using cold radioimmunoprecipitation assay (RIPA) buffer as described in the corresponding sections.

In vivo model: rats (n = 7 animals per group) were fed with the standard chow alone (control) or supplemented with EA, Uro-A, or Uro-B [72 mg kg^{-1} day^{-1}; human equivalent dose (HED): 813 mg for a 70 kg person]. In addition, rats were fed with a low dose of pomegranate extract (LPE; 21.6 mg kg^{-1} day^{-1}; HED: 0.24 g) or a high dose of pomegranate extract (HPE, 216 mg kg^{-1} day^{-1}; HED: 2.4 g) of PE. Dietary interventions lasted for 5 days. At the end of the experimental period, the animals were anaesthetized and sacrificed by decapitation. Samples of colon lumen contents, small intestine, kidney, and liver were taken immediately after slaughter, frozen in liquid nitrogen, and stored at −80 °C for metabolite determination and RNA extraction (n = 3 animals per treatment). The colon mucosa was also scraped off for RNA (n = 4 animals per treatment) or protein extraction (n = 3 animals per treatment) as described below.

RNA Isolation and Characterization. Total RNA was isolated using an RNeasy mini kit (Quiagen). RNA purity was characterized by spectrophotometry. Only samples with a ratio Abs_{260}/Abs_{280} between 1.8 and 2.1 were used for microarray experiments. The integrity of the rRNA was further checked using agarose gel electrophoresis (1%). Treatments and extractions were performed in triplicate.

Screening of mRNA by Microarray Analysis. A search for potential candidate genes for which transcription levels may have been modulated following exposure to EA and/or the urolithins was performed using Affymetrix microarray analysis (GeneChip Affymetrix microarray HG U133 Plus 2.0), as previously described (*20*). Microarray data were RMA-normalized and tested for differential gene expression using a t test, and FDR adjustment for multiple testing was implemented with software Gene Expression Pattern Analysis Suite (GEPAS, http://gepas.bioinfo.cipf.es/). To identify significantly altered biological pathways and top-regulated functions associated with responsive genes, Ingenuity Pathways Analysis (IPA, http://www.ingenuity.com) was used (*20*).

Validation of Gene Expression by Quantitative Reverse Transcription-Polymerase Chain Reaction (RT-PCR). Changes in the expression of five responsive genes were further assessed by one-step quantitative RT-PCR (Taqman system, Applied Biosystems, ABI, Madrid, Spain), as described elsewhere (*20*). RT-PCR reactions were performed in aliquots of the same RNA samples used for microarray screening and in new RNA samples prepared from independent experiments. Primers and probes for each gene were selected from those assays-on-demand available from ABI [cytochrome P450, family 1, subfamily A, polypeptide 1 (*CYP1A1*), Hs00153120_m1 (human) and Rn00487218_m1 (Rat); sulfotransferase family, cytosolic, 1A, phenol preferring, member 2 (*SULT1A2*), Hs00236900_m1; sulfotransferase family, cytosolic, 2A, dehydroepiandrosterone (DHEA) preferring, member 1 (*SULT2A1*), Hs00234219_m1; UDP glucuronosyltransferase 1 family, polypeptide

A10 (*UGT1A10*), Hs02516990]. All RT-PCR assays for a particular gene were undertaken at the same time under identical conditions and carried out in triplicate. The expression levels of target genes were normalized to the levels of the human *GAPDH* (Hs99999905_m1) and rat *GAPDH* (Rn99999916_s1) using a standard curve method for quantification.

Western Blot Analysis. Colon mucosa samples were homogenized in cold RIPA buffer containing protease inhibitor cocktail (Roche, Mannheim, Germany). Lysates were centrifuged at 15000*g* for 20 min at 4 °C, and the protein concentration was measured using the Bradford's reagent. A total of 40 μg of protein were subjected to 10−15% sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE) and transferred to polyvinylidene difluoride membranes (GE Healthcare, Buckinghamshire, U.K.) by electroblotting. To determine CYP1A1, membranes were incubated for 2 h with the primary antibody (Santa Cruz Biotechnology, Santa Cruz, CA) and 1 h with the secondary antibody conjugated to horseradish peroxidase (Sigma-Aldrich). Proteins were detected using an ECL plus detection system (GE Healthcare) according to the instructions of the manufacturer. For quantification, the density of the bands was determined with scanning densitometry, using the Image-Quant TL v2005 (GE Healthcare). Consistency in protein loading was controlled by probing stripped blots for GAPDH (Affinity BioreagentsTM, Golden, CO). Blots were carried out in triplicate ($n = 3$ animals per treatment).

Enzymatic Activity. Cells were plated in 24-well plates (3×10^4 cells per well), allowed to grow to confluence, and treated for 24, 48, and 72 h with EA, Uro-A, Uro-B, or the MIX at the concentrations stated above. CYP1 enzymatic activity was measured on living Caco-2 cells with a 7-ethoxy-resorufin-*O*-deethylase (EROD) assay (*23*). The fluorescence of the resorufin generated was measured in the culture media using a BMG Fluostar galaxy fluorescence microplate reader (MTX Lab Systems, Inc., Vienna, VA) with excitation at 520 nm and emission at 590 nm. Values are presented as nanomoles of resorufin formed per minute per well and normalized to the percentage of proliferating cells with respect to the control cells.

Changes in the activity of phase II enzymes induced in Caco-2 cells by the MIX were investigated using LC−MS/MS analysis. Cells were exposed for 72 h to the MIX at the concentrations indicated above. After the treatment, control (exposed to DMSO alone) and treated cells were washed with PBS and incubated with 30 μM quercetin for 0.5, 2, 4, 6, 8, 18, and 24 h to determine the formation of quercetin-conjugated metabolites. At the end of each time point, media was collected, acidified with formic acid (1.5%), and filtered through a pre-activated C-18 Sep-Pak cartridge (Millipore Corp.). Samples were eluted with 1 mL of MeOH, concentrated to dryness under N_2 flow at room temperature, and resuspended in 250 μL of MeOH prior to centrifugation (3000*g*, 5 min), dilution with Milli-Q water (1:1), and injection into the LC−MS/MS.

Sample Processing for the Determination of Phenolic Compounds and Metabolites. A total of 10 mg of PE was dissolved in 1 mL of water/formic acid (99:1) and filtered through a 0.45 μm membrane filter Millex-HV_{13} (Millipore Corp., Bedford, MA), and a 10 μL aliquot was analyzed by LC−MS/MS to determine the polyphenol content. Colon lumen contents and samples from liver and kidney (1 g each) were processed according to Espín et al. (*16*), and a 5 μL aliquot was analyzed by LC−MS/MS to identify and quantify the different metabolites.

LC−MS/MS Analysis. PE and samples from cell culture media and cell lysates (cellular uptake experiments) were analyzed in an 1100 series HPLC−DAD−MS−MS (Agilent Technologies, Waldbronn, Germany) following a published method (*24*).

Tissue extracts and cell culture media (phase II activity assays) were analyzed in a 1200 series HPLC−DAD system (Agilent Technologies, Waldbronn, Germany) equipped with an HTC Ultra mass detector in series (Bruker Daltonics, Bremen, Germany). The mass detector was an ion-trap mass spectrometer equipped with an electro-spray ionization (ESI, capillary voltage, 4 kV; nebulizer, 15 psi; dry gas, 5 L/min; dry temperature, 350 °C) system. Mass scan (MS) and MS/MS daughter spectra were measured from m/z 100 to 1200 using the Ultra scan mode (26000 m/z per second). Collision-induced fragmentation experiments were performed in the ion trap using helium as the collision gas, and the collision energy was set at 50%. Mass spectrometry data were acquired in the negative ionization mode. Chromatographic separations were carried out on a 150 × 0.5 mm inner diameter, 5 μm, reverse-phase SB C18 Zorbax column (Agilent, Waldbronn, Germany) using water/formic acid (99:1, v/v) (A) and acetonitrile (B) as the mobile phases at a flow rate of 10 μL/min. For tissue samples, the gradient started with 1% B in A to reach 60% B at 30 min, 90% B at 30 min for 5 min, and returning to the initial conditions (1% B). For culture media samples, the gradient started with 1% B in A to reach 50% B at 25 min, 90% B at 32 min for 5 min, and returning to the initial conditions (1% B). Chromatograms were recorded at 280, 305, and 360 nm.

Identification and quantification of quercetin, EA, punicalagin, Uro-A, and Uro-B were carried out by direct comparison (UV spectra and MS) with pure standards. Other ETs (punicalagin-like spectra compounds) and EA derivatives found in the PE were quantified as punicalagin (360 nm) and EA (360 nm), respectively. Urolithin-like metabolites and quercetin-derived conjugates were tentatively identified on the basis of their absorption and MS spectra as well as MS/MS fragments and quantified at 305 nm using Uro-A as a standard (*16*) and at 360 nm using quercetin as a standard, respectively.

Statistical Analysis. Gene and protein expression levels as well as activity results are presented as mean values ± standard deviation (SD) (at least $n = 3$). Differences between groups (control versus treated) were compared using an unpaired Student's t test (except for microarray analysis; see specific section). Results with a two-sided p value of < 0.05 or < 0.01 were considered statistically significant.

RESULTS

Xenobiotic-Metabolism-Related Gene Expression Changes in Caco-2 Cells Induced by EA and Urolithins. A full report of the microarray analysis and the genes significantly modulated in Caco-2 cells after 72 h exposure to EA and/or urolithins has been published elsewhere (*20*). Among other cell functions, analysis of resulting data with IPA identified changes in genes associated with the metabolism of small biomolecules. A more specific search revealed that the expression levels of some phase I and phase II xenobiotic-metabolism-related genes were regulated by the treatments (**Table 1**). Within the phase I enzymes, the mRNA levels of *CYP1A1* were upregulated in Caco-2 cells, especially in those cells treated with the MIX of compounds and in cells treated with Uro-B (> 25 fold change). In addition, Uro-B also induced the mRNA levels of *CYP1B1* and *CYP27B1* and downregulated *CYP3A5*. These effects were also seen in cells treated with the MIX or Uro-A, although the results were not found to be significant by the microarray statistical analysis (*20*). A group of transcripts representative of some phase II enzymes also exhibited significant changes in the treated cells. Some UGTs, specifically *UGT1A10* and *UGT1A6*, were induced after exposure to the MIX or the individual urolithins. Another two glucuronosyltransferases, *UGT2B15* and *UGT2B28*, were also significantly downregulated by the treatment with Uro-B. The MIX and Uro-A exerted similar effects on these two genes, although the results were not significant. There was an apparent general downregulation of sulfotransferases in cells treated with EA and/or urolithins. *SULT1A1*, *SULT1A2*, *SULT1A3*, *SULT2A1*, and *SULT1C1* were all significantly downregulated after exposure to the MIX or Uro-B, whereas EA or Uro-A also induced some of the changes (although they were not always significant). The relative expression changes of *CYP1A1*, *UGT1A10*, *SULT1A1*, and *SULT1A2* were further validated by RT-PCR (**Figure 1**). The induction of *CYP1A1* by Uro-A, Uro-B, and the MIX of compounds were confirmed by RT-PCR; however, the RT-PCR analysis did not detect any changes in the expression levels of this gene when the cells were treated with EA. The induction of *UGT1A10* was also confirmed in cells treated with Uro-A, Uro-B, and the MIX. Although the microarray analysis did not detect a change in the expression of this gene in cells treated with EA, the RT-PCR analysis showed a clear

Table 1. Differential Expression by Affymetrix Microarrays of Some Xenobiotic-Metabolism-Related Genes in Caco-2 Cells after 72 h Exposure to EA (10 μM), Uro-A (40 μM), Uro-B (40 μM), or a Mixture of These Compounds, MIX (10 μM EA + 40 μM Uro-A + 40 μM Uro-B)

			fold change			
Affymetrix probe	gene symbol	gene name	MIX	EA	Uro-A	Uro-B
		Phase I Enzymes				
		Cytochrome P450				
205749_at	*CYP1A1*	cytochrome P450, family 1, subfamily A, polypeptide 1	27.0[a]	2.5[b]	2.6[a]	25.3[a]
202436_s_at, 202437_s_at	*CYP1B1*	cytochrome P450, family 1, subfamily B, polypeptide 1	1.4[b]			2.0[a]
205676_at	*CYP27B1*	cytochrome P450, family 27, subfamily B, polypeptide 1	1.3[b]		1.3[b]	1.2[a]
214235_at, 214234_s_at, 205765_at	*CYP3A5*	cytochrome P450, family 3, subfamily A, polypeptide 5	−1.7[b]			−1.7[a]
		Phase II Enzymes				
		Transferases				
204532_x_at, 207126_x_at, 208596_s_at, 215125_s_at	*UGT1A10*	UDP glucuronosyltransferase 1 family, polypeptide A10	1.6[a]		1.4[a]	1.5[a]
232654_s_at, 206094_x_at	*UGT1A6*	UDP glucuronosyltransferase 1 family, polypeptide A6	1.6[a]		1.4[b]	1.6[a]
207392_x_at	*UGT2B15*	UDP glucuronosyltransferase 2 family, polypeptide B15	−2.3[b]		−1.8[b]	−2.0[a]
211682_x_at	*UGT2B28*	UDP glucuronosyltransferase 2 family, polypeptide B28	−1.7[b]		−1.7[b]	−1.9[a]
203615_x_at	*SULT1A1*	sulfotransferase family, cytosolic, 1A, phenol preferring, member 1	−1.8[a]	−1.4[b]		−1.3[a]
207122_x_at	*SULT1A2*	sulfotransferase family, cytosolic, 1A, phenol preferring, member 2	−1.6[a]	−1.5[a]		−1.3[a]
209607_x_at	*SULT1A3*	sulfotransferase family, cytosolic, 1A, phenol preferring, member 3 /	−1.8[a]	−1.4[b]		−1.2[a]
206292_s_at	*SULT2A1*	sulfotransferase family, cytosolic, 2A, dehydroepiandrosterone (DHEA) preferring, member 1	−4.4[a]	−2.0[b]	−1.3[b]	−1.7[a]
211470_s_at, 205342_s_at	*SULT1C1*	sulfotransferase family, cytosolic, 1C, member 1	−1.6[b]		−1.4[b]	−1.9[a]

[a] Significant changes. [b] No significant changes (*18*).

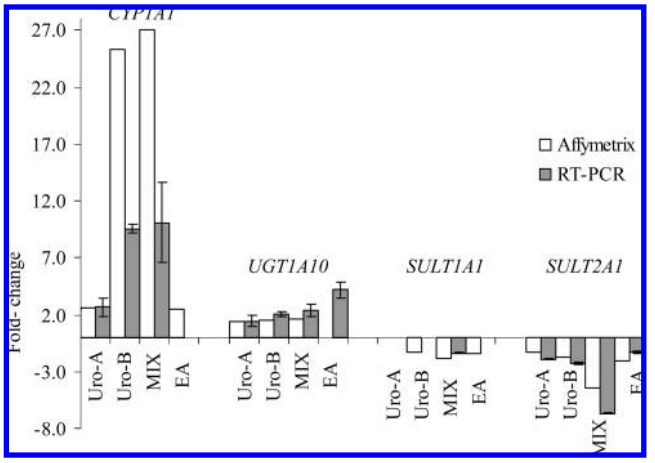

Figure 1. Expression plot of selected phase I and phase II genes with Affymetrix microarray and real-time RT-PCR analysis in Caco-2 cells exposed to Uro-A (40 μM), Uro-B (40 μM), EA (10 μM), or a mixture of these compounds MIX (10 μM EA + 40 μM Uro-A + 40 μM Uro-B) for 72 h. Fold- change values with respect to control cells (treated with DMSO alone) were normalized to GAPDH and are presented as mean values ± SD (n = 3). Missing bars indicate no change detected (fold change close to 1.0).

induction of *UGT1A10* by this compound. The downregulation of *SULT2A1* was verified in all of the treatments, but the downregulation of *SULT1A1* was only confirmed after treatment with the MIX of compounds.

Phase I and Phase II Enzyme Activity Changes Modulated by EA and Urolithins in Caco-2 Cells. Caco-2 cells were treated with EA and/or urolithins and assayed for ethoxyresorufin conversion after 24, 48, and 72 h of exposure. After treatment, the concentration of resorufin in the culture media was significantly increased after 72 h exposure to Uro-A ($p < 0.05$), Uro-B ($p < 0.01$), and the MIX ($p < 0.01$) (**Figure 2**). The highest induction was observed in cells treated with the MIX (15-fold increase over control cells), whereas Uro-A and Uro-B increased the levels of

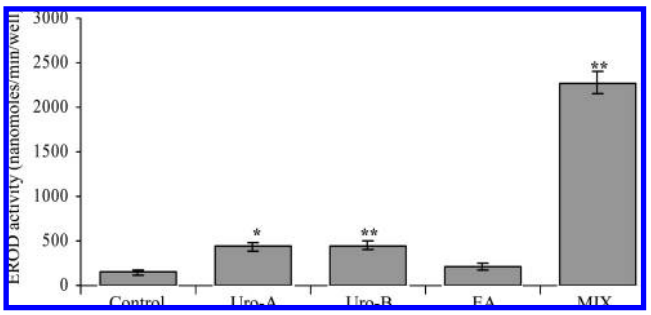

Figure 2. EROD activity in Caco cells exposed to Uro-A (40 μM), Uro-B (40 μM), EA (10 μM), or a mixture of these compounds MIX (10 μM EA + 40 μM Uro-A + 40 μM Uro-B) for 72 h. Control cells were exposed to DMSO alone. Values are the mean ± SD (n = 3). Asterisks denote significant differences from the control at (*) $p < 0.05$ and (**) $p < 0.01$.

resorufin by 2.9- and 3.0-fold, respectively. The pattern of induced EROD activity was similar to the pattern of *CYP1A1* mRNA expression. It is of note that Caco-2 cells were able to form small quantities of Uro-A from Uro-B (results not shown), which may be mediated by the induced CYP1 hydroxylase activity.

To investigate the effects of EA and the urolithins on phase II enzyme activity, Caco-2 cells were treated with DMSO (control) or the MIX for 72 h, followed by incubation with quercetin to monitor the formation of quercetin conjugates. LC−MS/MS analysis revealed the synthesis and release to the culture media of several quercetin derivatives in the control cells (**Figure 3a**), which were all also detected in the treated cells. The major conjugate formed was methyl-quercetin (**5**) followed by methyl-quercetin sulfate (**6**), methyl-quercetin glucuronide (**2**), quercetin glucuronide (**1**), and quercetin sulfate (**3**). The extent of the production of these conjugates was assessed over a 24 h period (**Figure 3b**), after which approximately 80% of the initial quercetin (**4**) had disappeared from the culture media in both the control and treated cells. The most marked differences between the control and

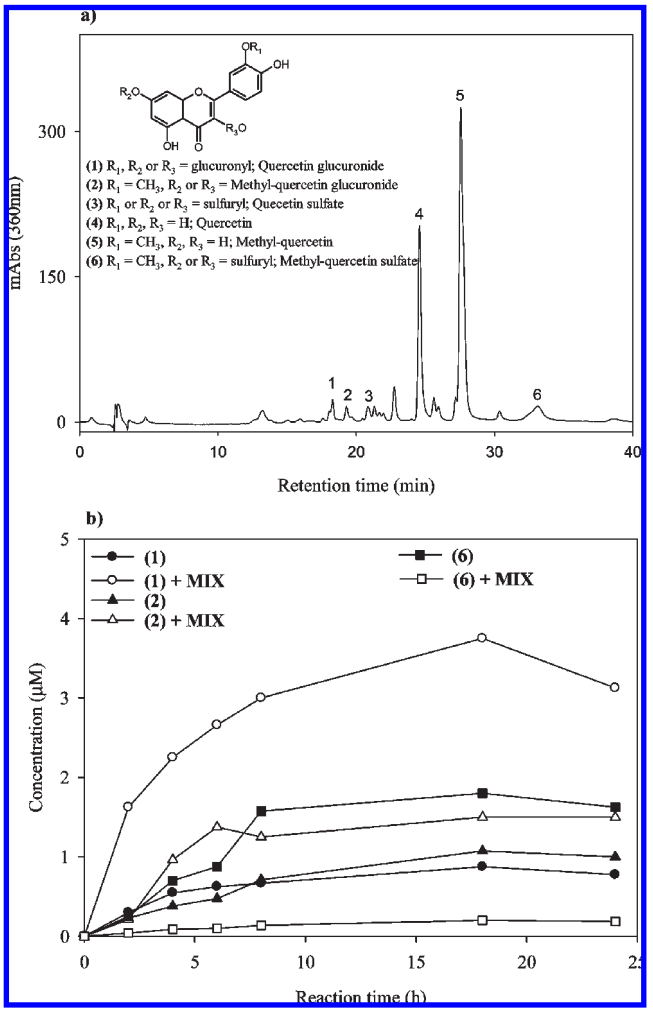

Figure 3. Phase II enzyme activity in Caco-2 cells exposed to a mixture of 10 μM EA + 40 μM Uro-A + 40 μM Uro-B (MIX) for 72 h. (a) Chemical structures of quercetin and conjugates and chromatographic profile (360 nm) of cell culture media from control cells after 8 h of incubation with quercetin (**4**). Metabolites detected: quercetin glucuronide (**1**), methyl-quercetin glucuronide (**2**), quercetin sulfate (**3**), methyl-quercetin (**5**), and methyl-quercetin sulfate (**6**). (b) Changes in the concentration of metabolites throughout the incubation time: solid symbols (culture media from control cells) and open symbols (culture media from cells treated with the MIX).

treated cells were those found for quercetin glucuronide (**1**), whose production over the time period examined was ~4-fold higher in the treated cells than in the control cells. An induction of ~1.5-fold in the synthesis of methyl-quercetin glucuronide (**2**) was also measured. This increase in the glucuronidation rate may be associated with the induction of *UGT1A10* expression. The production of methyl-quercetin sulfate (**6**) was decreased by ~9-fold in the treated cells, which may be a consequence of the observed lower expression of SULTs.

Caco-2 Cell Metabolism of EA and Uro-A and Uro-B. We further examined whether EA and urolithins were taken up and metabolized into any conjugated derivatives by the Caco-2 cells. For details of the compounds and their metabolites, see **Figure 4** and **Table 2**. After incubation of the cells with Uro-A (**9**), LC−MS/MS analysis of the culture media (at any of the time points tested) detected the formation mostly of Uro-A-glucuronide (**7**) as well as small quantities of Uro-A-sulfate (**8**) and Uro-A-disulfate (**10**). Incubation of Caco-2 cells with Uro-B (**13**) led to the formation and excretion of Uro-B-glucuronide (**11**) and trace quantities of Uro-B-sulfate (**12**). It was also possible to detect small amounts of Uro-A (**9**) and Uro-A-glucuronide (**7**). When the cells were exposed to the MIX of urolithins and EA (**14**), the major metabolites detected were the glucuronides of Uro-A (**7**) and Uro-B (**11**) as well as the Uro-A glucuronide sulfate conjugate (**15**). We also detected the formation of small quantities of the mono- and dimethyl derivatives of EA (**16** and **17**). All of the compounds and their conjugates were also detected and identified in the cell lysates, confirming the entrance of the compounds into the cells, conjugation, and excretion to the culture media.

Effect of EA and Urolithins on CYP1A1 in the Rat. *In vitro* results demonstrated that EA and urolithins exerted the most significant induction on CYP1A1 in Caco-2 cells, especially when the cells were exposed to the MIX or Uro-B for 72 h. In the following set of experiments, we investigated the expression of *CYP1A1* in the rat colon mucosa after *in situ* incubation with EA and/or the urolithins. The incubation time was reduced to a 2 h period to limit the disturbance effects caused by the surgical procedure. To check whether the induction of *CYP1A1* in Caco-2 cells occurred already at early time points, we performed a time course study. After exposure to Uro-A, Uro-B, and the MIX, the $t_{1/2}$ values obtained were 4.6, 3.2, and 3.8 h and the maximum fold induction was 2.6-, 2.8-, and 6-fold, respectively. mRNA levels of *CYP1A1* were augmented as early as 2 h after the beginning of treatment with the MIX of compounds, which suggested that the 2 h *in situ* incubation was long enough to detect changes in the expression of this gene in the rat colon mucosa. *In situ* incubation with Uro-A (40 μM), Uro-B (40 μM), or the MIX of the compounds (40 μM Uro-A + 40 μM Uro-B + 10 μM EA) dissolved in PBS confirmed the induction of *CYP1A1* expression in the rat colon mucosa by 3.2 ± 1.3 (GAPDH normalized fold change mean value ± SD; n = 4), 7.8 ± 1.8, and 3.7 ± 0.4, respectively. In addition, EA (10 μM) in PBS also induced the mRNA levels of *CYP1A1* (4.3 ± 2.3) in the rat colon. However, when the *in situ* incubations were repeated with the compounds dissolved in sunflower oil, no significant changes in the expression levels of *CYP1A1* were detected (normalized fold change values close to 1.0). In rats fed with a standard diet supplemented with each individual compound or with a low or high dose of pomegranate extract, no significant induction of *CYP1A1* mRNA was observed in the colon mucosa. We also assessed protein lysates from rat colonic mucosa by Western Blots in both the *in situ* and *in vivo* experiments (**Figure 5**). We found that, *in situ*, the protein levels of CYP1A1 were slightly increased by Uro-A and Uro-B and significantly augmented ($p < 0.05$) after exposure to the MIX (in PBS). In the *in vivo* model, no significant differences in the levels of CYP1A1 in the colon mucosa were detected between the control group and any of the treated groups.

We determined the presence and quantity of EA and/or urolithins in the colon after the consumption for 5 days of EA, Uro-A, Uro-B, or HPE. LC−MS/MS analysis of the colon contents revealed that, after the intake of EA, the major metabolite detected was Uro-A (concentration range detected, 6−168 μM) (**9**), followed by Uro-B (0.5−20 μM) (**13**), Uro-C (m/z^- 243; 4−12 μM) (**18**), and minor quantities of EA (up to ~6 μM) (**14**). When the animals were fed with Uro-A, the only compound detected was the Uro-A (32−200 μM) (**9**), and after the ingestion of Uro-B, we were able to detect Uro-B (20−72 μM) (**13**) and small quantities of Uro-A (1−10 μM) (**9**). The PE used in this study contained 35% punicalagins (three isomers, m/z^- 1083), 13% punicalin (m/z^- 781), 4.5% EA glucoside (m/z^- 463), and 8.9% free EA. Other ellagitannins with punicalagin-like UV spectra and MS/MS at m/z^- 301 were also detected. Consumption of a high dose of

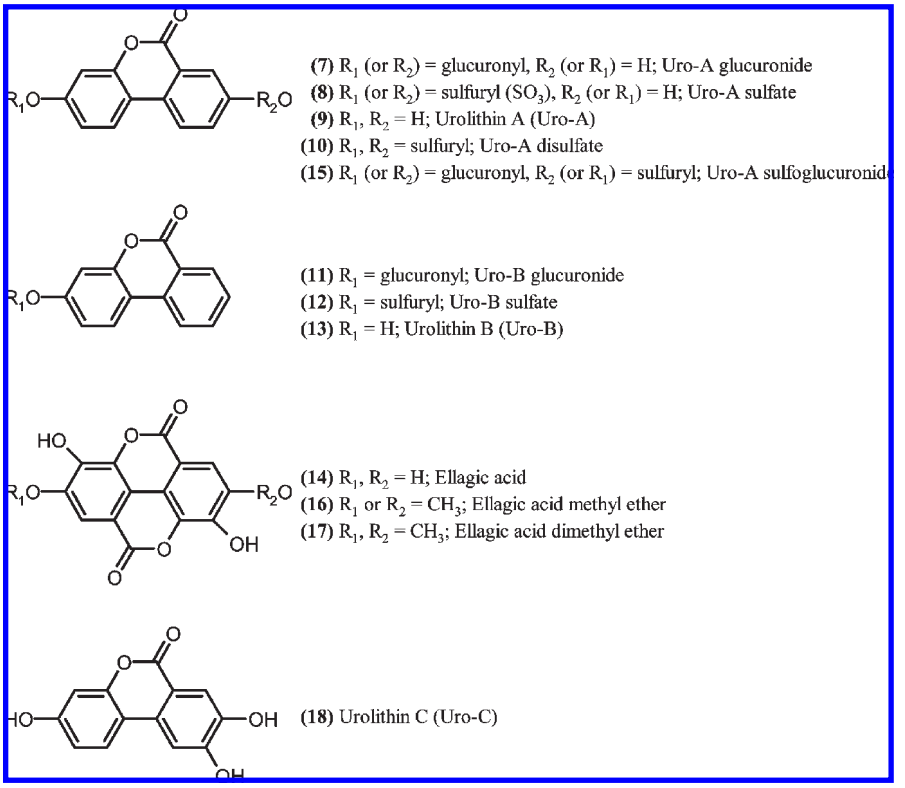

Figure 4. Chemical structures of EA, Uro-C, Uro-A, Uro-B, and their respective detected conjugates.

Table 2. Quercetin and EA Metabolites Detected in Caco-2 Cells and the Rat Colon

structure	compound number	Rt (min)	MS (M − H)	MS/MS
		Quercetin Metabolites		
quercetin glucuronide	**1**	18.3[a]	477	301, 175
methyl-quercetin glucuronide	**2**	19.2[a]	491	315, 175
quercetin sulfate	**3**	20.9[a]	381	301
quercetin	**4**	24.5[a]	301	179, 151
methyl-quercetin	**5**	27.8[a]	315	301
methyl-quercetin sulfate	**6**	33.3[a]	395	301
		Ellagic Acid Metabolites		
Uro-A glucuronide	**7**	12.8[b]	403	227, 175
Uro-A sulfate	**8**	18.5[b]	307	227
Uro-A	**9**	19.6[b], 23[c]	227	210, 199, 183, 160
Uro-A disulfate	**10**	21.8[b]	387	307, 227
Uro-B glucuronide	**11**	17[b]	389	211, 175
Uro-B sulfate	**12**	21.1[b]	291	211
Uro-B	**13**	25[b], 28.2[c]	211	167
ellagic acid	**14**	14.8[b], 17.8[c]	301	257, 229
Uro-A sulfoglucuronide	**15**	16.5[b]	483	307, 227, 175
ellagic acid methyl ether	**16**	18[b]	315	300
ellagic acid dimethyl ether	**17**	21.8[b]	329	315, 300
Uro-C	**18**	21.1[c]	243	199, 183, 163

[a] LC−MS/MS analysis of quercetin and its derived metabolites in the cell culture media as described in the Materials and Methods. [b] LC−MS/MS method for the analysis of EA and metabolites in the cellular uptake experiments (*24*). [c] LC−MS/MS method for the analysis of EA and metabolites in the tissue samples as described in the Materials and Methods.

PE led to the detection in the colon of Uro-A (7−34 μM) (**9**), Uro-B (2−65 μM) (**13**), Uro-C (1.5−5.4 μM) (**18**), and trace quantities of EA (**14**).

The expression levels of *CYP1A1* were also analyzed in the liver, kidney, and fragments of the small intestine from the control animals (fed with the standard chow) and from animals supplemented with EA, Uro-A, Uro-B, or PE. Results are presented in **Figure 6**. Although *CYP1A1* was expressed in all of the organs tested, the highest levels of expression were found in the small intestine followed by the kidney, whereas the colon mucosa and the liver exhibited the lowest mRNA levels of *CYP1A1*. The expression of *CYP1A1* was significantly induced in the kidney of rats that had consumed the pomegranate extract ($p < 0.05$) and in the liver upon consumption of the HPE or Uro-A ($p < 0.05$). LC−MS/MS analysis of the kidney and liver extracts detected the presence of trace quantities of the Uro-A-glucuronide (**7**) in the kidney and liver from animals fed with the pomegranate extract, whereas in the group of animals fed with Uro-A, we were able to quantify the Uro-A-glucuronide (**7**) (0.12−0.20 μg/g of tissue) as well as to detect trace quantities of Uro-A.

DISCUSSION

The colon epithelium constitutes a first line of defense against orally ingested xenobiotics (carcinogens, drugs, natural compounds, etc.) by virtue of being equipped with phase I and phase II activating and/or detoxifying enzymes. Regulation of the levels and activity of these enzymes may contribute to prevent colon cancer development by enhancing detoxification of carcinogens (*1*, *2*). It may also affect the bioavailability of drugs with potential adverse effects for human health (*25*). Regular consumption of polyphenol-rich foods can supply micromoles of these compounds and their physiological derivatives to the large intestine (*26*), where they might exert some of their reported anticancer effects by mechanisms not yet fully understood. One hypothesis is that these compounds may exert modulatory effects on phase I and phase II enzymes and regulate the metabolism of carcinogens and drugs. In the present investigation, we have shown that the polyphenol EA and its colon metabolites, urolithins, at concentrations that

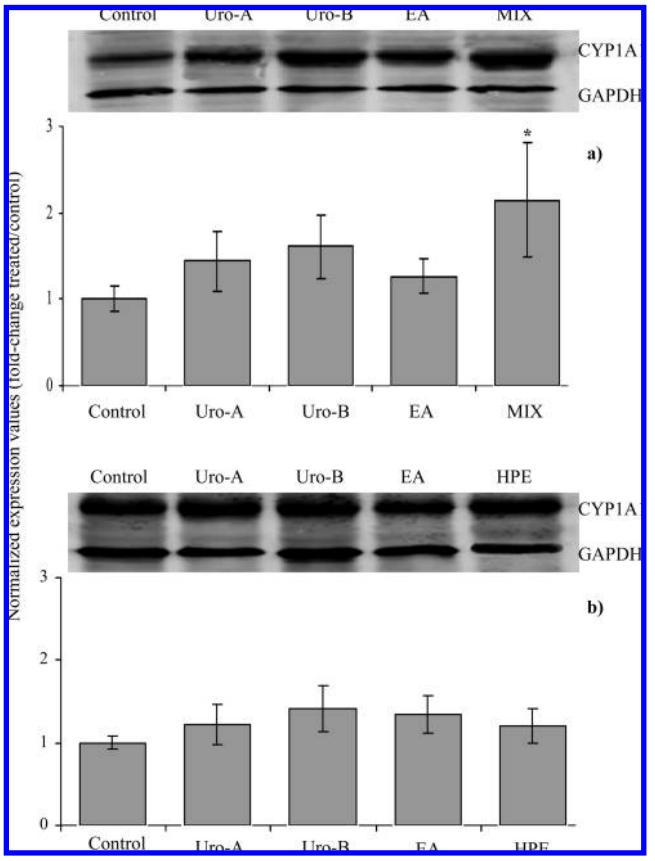

Figure 5. CYP1A1 immunoblot analysis of rat colon mucosa lysates from *in situ* and *in vivo* models. (a) *In situ* experimental conditions: the rat colon was incubated with Uro-A (40 μM), Uro-B (40 μM), EA (10 μM), or the MIX (10 μM EA + 40 μM Uro-A + 40 μM Uro-B) in PBS for 2 h. The control group was exposed to PBS alone. (b) *In vivo* experiment: animals were fed with the standard chow alone (control) or supplemented with EA, Uro-A, or Uro-B (72 mg kg^{-1} day^{-1}; HED: 813 mg for a 70 kg person) and with a high dose of pomegranate extract (HPE, 216 mg kg^{-1} day^{-1}; HED: 2.4 g). All of the dietary interventions lasted for 5 days. Protein expression is presented as the normalized (to GAPDH) fold change between treated and control mucosa. Bars indicate the mean value ± SD (n = 3). An asterisk denotes significant differences from the control at (*) $p < 0.05$.

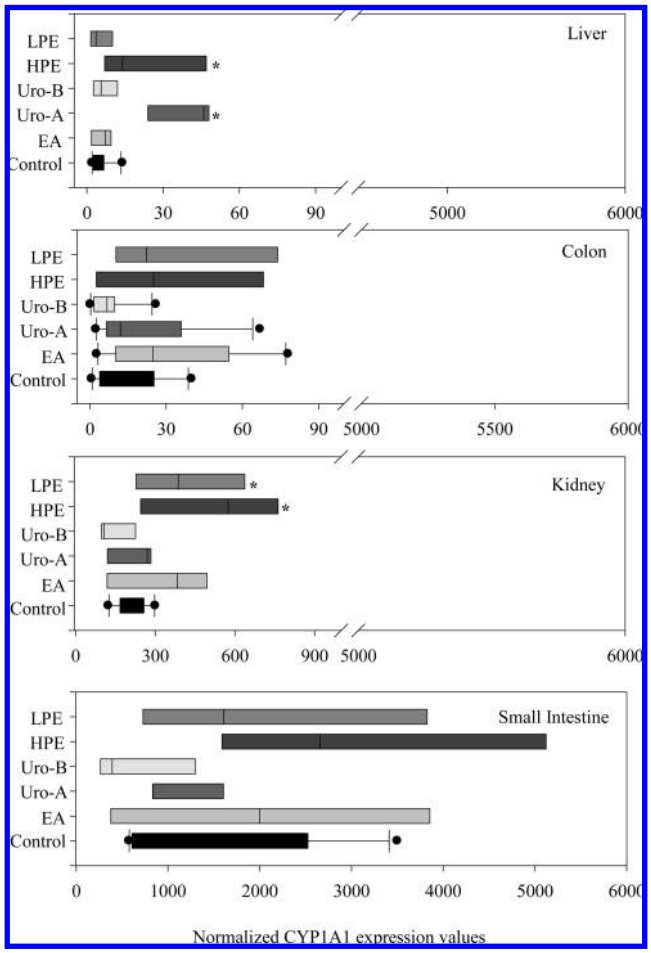

Figure 6. Box and whisker plots displaying the distribution of the organ-specific relative expression levels of *CYP1A1* mRNA in rats fed with the standard chow alone or supplemented with EA, Uro-A, or Uro-B (72 mg kg^{-1} day^{-1}; HED: 813 mg for a 70 kg person) and a low dose (LPE, 21.6 mg kg^{-1} day^{-1}; HED: 0.24 g) or a high dose (HPE, 216 mg kg^{-1} day^{-1}; HED: 2.4 g) of pomegranate extract. The horizontal line in the boxes indicate the median. The whiskers display the range. All of the dietary interventions lasted for 5 days. Expression values are normalized to GAPDH (n = 3−9). An asterisk denotes significant differences from the control at (*) $p < 0.05$.

are achievable in the colon from the diet, are able to specifically modulate the mRNA levels of several CYPs in Caco-2 cells. Only a few genes from the 53 CYP human genes represented on the microarray chip exhibited significant changes after exposure to the compounds. In particular, CYP1A1 and CYP1B1 were upregulated in the treated cells, especially CYP1A1. These cytochromes are involved in the metabolism of many carcinogenic PAHs (*2*), and their inhibition has been reported to suppress tumor formation caused by PAHs (*27*). In this context, the induction of these genes by EA and urolithins in the colon cells may be interpreted as a harmful effect. However, recent work has indicated that, whereas CYP1B1 plays an important role in activating PAH procarcinogens, CYP1A1 is more involved in detoxification (*8*), suggesting that the potent specific induction of CYP1A1 in the colon cells by EA and urolithins (> 25-fold) may have a protective effect against some oral carcinogens. In addition, two other CYPs were regulated in the Caco-2 cells after exposure to each of the urolithins or the MIX of compounds. The CYP27B1 or calcidiol 1-monooxygenase involved in the synthesis of the active metabolite of the vitamin D, 1,25-diOH vitamin D3 (1,25-D3) (*28*), was slightly upregulated by the urolithins. 1,25-D3 is induced in colonocytes by phytoestrogens, such as genistein, and this has been suggested to exert a protective role against colon tumor growth (*29*). CYP3A5, a cytochrome implicated in the metabolism of anticancer drugs (*30*), was significantly downregulated by Uro-B. Recently, it has been reported that the fungicide imazalil induces CYP1A1 and inhibits CYP3A4 activity in Caco-2 cells (*25*). The authors postulate that co-ingestion of this food contaminant with CYP1A1- or CYP3A4-metabolizable drugs has the potential to interfere with the drug bioavailability with possible adverse effects for human health. It does appear that regulation of CYPs can have beneficial and/or detrimental effects. The response is CYP-, substrate-, and molecule co-ingestion-dependent.

The metabolic fate of xenobiotics also depends upon phase II enzyme activity. It is generally accepted that the induction of glucuronidation by UGTs facilitates detoxification of potential colon carcinogens (*9*). Some UGTs are specifically induced in Caco-2 cells by polyphenols; e.g., quercetin is able to induce *UGT1A6* (*31*), whereas the flavonoid chrysin induces the expression of *UGT1A1* but has no effect on

UGT1A6 (*32*). In the present study, we have shown that *UGT1A10* is specifically induced in Caco-2 cells after exposure to the urolithins. *UGT1A10* has been identified as the major UGT that conjugates PAHs, and thus, its induction may exert chemoprotective effects (*33*). *UGT1A10* is also highly efficient in the glucuronidation of flavonoids. Human *UGT1A10* has been shown to form the 7-*O*-, 3-*O*-, 4′-*O*-, and 3′-*O*-quercetin glucuronides (*34*). We used quercetin as a substrate to determine the effect of the MIX treatment on the Caco-2 cell rate of glucuronidation, and we detected an increase in the synthesis of some quercetin glucuronide derivatives. *UGT1A10* is highly expressed in the lower GI tract, where it may play a role in conjugating aromatic structures formed by the microbiota (*35*). We have also shown that the microbiota EA metabolites, Uro-A and Uro-B, are extensively conjugated by Caco-2 cells predominantly via glucuronidation. These results are in good agreement with the observed induction of *UGT1A10*.

Caco-2 cell exposure to EA and urolithins also caused a downregulation of the expression of several cytosolic SULTs. These enzymes can sulfonate and detoxify many xenobiotic compounds. Some SULTs can also activate environmental carcinogens, and thus inhibition of these enzymes may have chemopreventive effects (*2*). The human SULT1A or phenol sulfotransferase (PST) subfamily has a preference for small phenols, including dietary flavonoids (*36*), many of which have been shown to selectively inhibit PST activity (*37*). Among the pomegranate polyphenols, EA has been shown to inhibit the activity of purified PST (*37*) and punicalagin also inhibits PST activity but not the expression of the SULT1A family of genes in Caco-2 cells (*38*). However, EA and punicalagin are metabolized in the intestine to form the urolithins and are unlikely to be present in the colon at the reported inhibitory concentrations. Our study shows a downregulation of the expression of the three members of the SULT1A subfamily in Caco-2 cells exposed to dietary levels of Uro-B and, more noticeably, when exposed to the MIX of compounds. In cells treated with the MIX of compounds, the decrease in SULT expression was accompanied by a reduction in the rate of sulfonation of methyl-quercetin, indicative of a lower sulfotransferase activity. Also, in Caco-2 cells incubated with Uro-A, which did not affect the expression of SULTs, we were able to detect the synthesis of the Uro-A sulfated derivatives, whereas in cells exposed to the MIX, which exhibited maximum downregulation of these enzymes, no sulfate derivatives were detected. Sulfonation also plays a major role in the metabolism and inactivation of estrogens. The urolithins exert some estrogenic activity in breast cancer cells, where these compounds are metabolized mostly by SULTs (*39*). However, in the colon cancer cells, glucuronidation of the urolithins is favored over sulfonation, suggesting that the detoxifying mechanisms of these compounds are tissue-specific.

Our next step was to try to confirm the observed *in vitro* CYP1A1 induction in a rat model. Colon *in situ* incubations of aqueous-buffer-dissolved Uro-A, Uro-B, or the MIX induced the levels of mucosal CYP1A1 at both the mRNA and protein levels. Even though there may be differences in the levels of metabolic enzymes between species, these results agree with the response of the human colon cells and indicate that the compounds can exert similar effects in the animal model. In contrast, when the compounds were dissolved in sunflower oil, no induction was observed. Because the oil did not affect the expression of CYP1A1 (results not shown), our results indicate a critical effect of the matrix in which the compounds are dissolved. We hypothesize that the oil may affect the diffusion of the molecules through the unstirred water layer adjacent to the mucosa and consequently the absorption of the molecules and their effects on the epithelium. As a further confirmation of the interferences caused by the food matrix, our *in vivo* experiments also showed a lack of CYP1A1 induction in the colon of rats fed with the different compounds, even though we detected significant quantities of these molecules present in the lumen. Food matrix interferences may be caused by physicochemical factors or interactions with other compounds present in the food.

Our results on organ-specific CYP1A1 expression were in agreement with previous published results (*40*). In addition to interindividual variations, differences between genders may explain, in part, the variability observed in our results because our different animal groups included both male and female rats. Despite the high variability of the data, we detected a slight but significant induction of CYP1A1 in the liver and kidney of animals fed with Uro-A or the pomegranate extract. It is noteworthy that, while in the colon, where the compounds were present at high concentrations, there was no effect on CYP1A1; induction of the expression of this gene could be seen in the liver or kidney, where only very small quantities of Uro-A and/or Uro-A glucuronide were detected. This result supports the neutralizing effect of the food matrix in the colon, which is absent in systemic tissues. Up to 31 different metabolites of the ellagitannins have been identified in the plasma, intestine, and bile from pigs (*16*). However, none of these metabolites was detected in the pig tissues, such as liver or kidney. Although we can not fully discard that some other conjugates of urolithins might reach some of the studied organs, it seems unlikely or the levels must be well below the detection limits. We cannot discard either that the observed induction in the liver or kidney may be due to other absorbed non-ellagitannin-derived components of the pomegranate extract.

Our results constitute a good example of the difficulty of reproducing *in vitro* results in *in vivo* studies. Some of the faults typically made in cell culture studies include the use of compounds or extracts that *in vivo* are not available to those cells or the use of unrealistic concentrations. For example, pomegranate juice (PJ) inhibits *in vitro* liver CYP3A activity (*41*), which suggested an effect on the metabolism of drugs that are substrates of this enzyme. However, PJ, as such a complex food, would never reach the liver, and thus far, *in vivo* studies have not demonstrated unequivocally that the consumption of PJ may interfere with the metabolism and clearance of some drugs (*42*). There is a need for establishing the actual metabolites and their concentrations *in vivo* as well as for investigating their effects using suitable cell models. We were careful to select an appropriate colon cell model and to use the metabolites found *in vivo* in the colon. Also, the concentrations of EA and urolithins used in the *in vitro* and *in situ* experiments were realistic and representative of those concentrations that can be reached in the colon after the intake of dietary quantities of these compounds or the natural fruit extracts containing the precursor ellagitannins and EA. Our results also indicate that other factors, such as the food matrix, have critical effects on the *in vivo* responses in the colon.

LITERATURE CITED

(1) Yu, S.; Kong, A. N. Targeting carcinogen metabolism by dietary cancer preventive compounds. *Curr. Cancer Drug Targets* **2007**, *7*, 416–424.

(2) Shimada, T. Xenobiotic-metabolizing enzymes involved in activation and detoxification of carcinogenesis polycyclic aromatic hydrocarbons. *Drug Metab. Pharmacokinet.* **2006**, *21*, 257–276.

(3) McKay, J. A.; Murray, G. I.; Weave, R. J.; Ewen, S. W.; Melvin, W. T.; Burke, M. D. Xenobiotic metabolizing enzyme expression in colonic neoplasia. *Gut* **1993**, *34*, 1234–1239.
(4) Turkey, R. H.; Strassburg, C. P. Human UDP-glucuronosyltransferases: Metabolism, expression and disease. *Annu. Rev. Pharmacol. Toxicol.* **2000**, *40*, 581–616.
(5) Kumarakulasingham, M.; Rooney, P. H.; Dundas, S. R.; Telfer, C.; Melvin, W. T.; Curran, S.; Murray, G. I. Cytochrome P450 profile of colorectal cancer: Identification of markers of prognosis. *Clin. Cancer Res.* **2005**, *11*, 3758–3765.
(6) Sivaraman, L.; Leatham, M. P.; Yee, J.; Wilkens, L. R.; Lau, A. F.; Le Marchand, L. CYP1A1 genetic polymorphisms and in situ colorectal cancer. *Cancer Res.* **1994**, *54*, 3692–3695.
(7) Conney, A. H. Enzyme induction and dietary chemicals as approaches to cancer chemoprevention: The seventh DeWitt S. Goodman lecture. *Cancer Res.* **2003**, *63*, 7005–7031.
(8) Uno, S.; Dalton, T. P.; Derkenne, S.; Curran, C. P.; Miller, M. L.; Shertzer, H. G.; Nebert, D. W. Oral exposure to dibenzo[*a,l*]pyrene in the mouse: Detoxication by inducible cytochrome P450 is more important than metabolic activation. *Mol. Pharmacol.* **2004**, *65*, 1225–1237.
(9) Moon, Y. J.; Wang, X.; Morris, M. E. Dietary flavonoids: Effects on xenobiotic and carcinogen metabolism. *Toxicol. In Vitro* **2006**, *20*, 187–210.
(10) Kale, A.; Gawande, S.; Kotwal, S. Cancer phytotherapeutics: Role for flavonoids at the cellular level. *Phytother. Res.* **2008**, *22*, 567–577.
(11) Pohl, C.; Will, F.; Dietrich, H.; Schrenk, D. Cytochrome P450 1A1 in Caco-2 cells: Modulation by apple juice extract and certain apple polyphenols. *J. Agric. Food Chem.* **2006**, *54*, 10262–10268.
(12) Hidaka, M.; Okumura, M.; Fujita, K.-I.; Ogikubo, T.; Yamasaki, K.; Iwakiri, T.; Setoguchi, N.; Arimori, K. Effects of pomegranate juice on human cytochrome P450 3A (CYP3A) and carbamazepine pharmacokinetics in rats. *Drug Metab. Dispos.* **2005**, *33*, 644–648.
(13) Nagata, M.; Hidaka, M.; Sekiya, H.; Kawano, Y.; Yamasaki, K. M.; Arimori, K. Effects of pomegranate juice on human cytochrome P4502C9 and tolbutamide pharne pharmacokinetics in rats. *Drug Metab. Dispos.* **2007**, *35*, 302–305.
(14) Faria, A.; Monteiro, R.; Azevedo, I.; Calhau, C. Pomegranate juice effects on cytochrome P450S expression: In vivo studies. *J. Med. Food* **2007**, *10*, 643–649.
(15) Cerdá, B.; Periago, P.; Espín, J. C.; Tomás-Barberán, F. A. Identification of urolithin A as a metabolite produced by human colon microflora from ellagic acid and related compounds. *J. Agric. Food Chem.* **2005**, *53*, 5571–5579.
(16) Espín, J. C.; González-Barrio, R.; Cerdá, B.; López-Bote, C.; Rey, A. I.; Tomás-Barberán, F. A. Iberian pig as a model to clarify obscure points in the bioavailability and metabolism of ellagitannins in humans. *J. Agric. Food Chem.* **2007**, *55*, 10476–10485.
(17) Seeram, N. P.; Adams, L. S.; Henning, S. M.; Niu, Y.; Zhang, Y.; Nair, M. G.; Heber, D. *In vitro* antiproliferative, apoptotic and antioxidant activities of punicalagin, ellagic acid and a total pomegranate tannin extract are enhanced in combination with other polyphenols as found in pomegranate juice. *J. Nutr. Biochem.* **2005**, *16*, 360–367.
(18) Larrosa, M.; Tomás-Barberán, F. A.; Espín, J. C. The dietary hydrolysable tannin punicalagin releases ellagic acid that induces apoptosis in human colon adenocarcinoma Caco-2 cells by using the mitochondrial pathway. *J. Nutr. Biochem.* **2006**, *17*, 611–625.
(19) Päivärinta, E.; Pajari, A. M.; Törrönen, R.; Mutanen, M. Ellagic acid and natural sources of ellagitannins as possible chemopreventive agents against intestinal tumorigenesis in the Min mouse. *Nutr. Cancer* **2006**, *1*, 79–83.
(20) González-Sarrías, A.; Espín, J. C.; Tomás-Barberán, F. A.; García-Conesa, M. T. Gene expression, cell cycle arrest and MAPK signalling regulation in Caco-2 cells exposed to acid ellagic and its metabolites, urolithins. *Mol. Nutr. Food Res.* **2009**, [Epub ahead of print] PMID: 19437480.
(21) Cerdá, B.; Llorach, R.; Cerón, J. J.; Espín, J. C.; Tomás-Barberán, F. A. Evaluation of the bioavailability and metabolism in the rat of punicalagin, an antioxidant polyphenol from pomegranate juice. *Eur. J. Nutr.* **2003**, *42*, 18–28.
(22) Larrosa, M.; Tomás-Barberán, F. A.; Espín, J. C. The dietary hydrolysable tannin punicalagin releases ellagic acid that induces apoptosis in human colon adenocarcinoma Caco-2 cells by using the mitochondrial pathway. *J. Nutr. Biochem.* **2006**, *17*, 611–625.
(23) Ciolino, H. P.; Bass, S. E.; MacDonald, C. J.; Cheng, R. Y. S.; Yeh, G. C. Sulindac and its metabolites induce carcinogen metabolizing enzymes in human colon cancer cells. *Int. J. Cancer* **2008**, *122*, 990–998.
(24) Cerdá, B.; Espín, J. C.; Parra, S.; Martínez, P.; Tomás-Barberán, F. A. The potent in vitro antioxidant ellagitannins from pomegranate juice are metabolised into bioavailable but poor antioxidant hydroxy-6*H*-dibenzopyran-6-one derivatives by the colonic microflora of healthy humans. *Eur. J. Nutr.* **2004**, *43*, 205–220.
(25) Sergent, T.; Dupont, I.; Jassogne, C.; Ribonnet, L.; van der Heiden, E.; Scippo, M. L.; Muller, M.; McAlister, D.; Pussemier, L.; Larondelle, Y.; Schneider, Y. J. CYP1A1 induction and CYP3A4 inhibition by the fungicide imazalil in the human intestinal Caco-2 cells—Comparison with other conazole pesticides. *Toxicol. Lett.* **2009**, *184*, 159–168.
(26) Kahle, K.; Kraus, M.; Scheppach, W.; Ackermann, M.; Ridder, F.; Richling, E. Studies on apple and blueberry fruit constituents: Do the polyphenols reach the colon after ingestion?. *Mol. Nutr. Food Res.* **2006**, *50*, 418–423.
(27) Chun, Y.-J.; Kim, S. Discovery of cytochrome P450 1B1 inhibitors as new promising anti-cancer agents. *Med. Res. Rev.* **2003**, *23*, 657–668.
(28) Seliskar, M.; Rozman, D. Mammalian cytochromes P450—Importance of tissue specificity. *Biochim. Biophys. Acta* **2007**, *1770*, 458–466.
(29) Kállay, E.; Adlercreutz, H.; Farhan, H.; Lechner, D.; Bajna, E.; Gerdenitsch, W.; Campbell, M.; Cross, H. S. Phytoestrogens regulate vitamin D metabolism in the mouse colon: Relevance for colon tumor prevention and therapy. *J. Nutr.* **2002**, *132*, 3490S–3493S.
(30) Martínez, C.; García-Marín, E.; Pizarro, R. M.; García-Gamito, F. J.; Agúndez, J. A. G. Expression of paclitaxel-inactiving CYP3A activity in human colorectal cancer: Implications for drug therapy. *Br. J. Cancer* **2002**, *87*, 681–686.
(31) Bock, K. W.; Eckle, T.; Ouzzine, M.; Fournel-Gigleux, S. Coordinate induction by antioxidants of UDP-glucuronosyltransferase UGT1A6 and the apical conjugate export pump MRP2 (multidrug resistance protein 2) in Caco-2 cells. *Biochem. Pharmacol.* **2000**, *59*, 467–470.
(32) Galijatovic, A.; Otake, Y.; Walle, K.; Walle, T. Induction of UDP-glucuronosyltransferase UGT1A1 by the flavonoid Chrysin in Caco-2 cells—Potential role in carcinogen bio-inactivation. *Pharm. Res.* **2001**, *18*, 374–379.
(33) Starlard-Davenport, A.; Lyn-Cook, B.; Radominska-Pandya, A. Novel identification of UDP-glucuronosyltransferase 1A10 as an estrogen-regulated target gene. *Steroids* **2008**, *73*, 138–147.
(34) Boersma, M. G.; van der Woude, H.; Bogaards, J.; Boeren, S.; Vervoort, J.; Cnubben, N. H. P.; van Iersel, M. L. P. S.; van Bladeren, P. J.; Rietjens, I. M. C. M. Regioselectivity of phase II metabolism of Luteolin and quercetin by UDP-glucuronosyl transferases. *Chem. Res. Toxicol.* **2002**, *15*, 662–670.
(35) Basu, N. K.; Kubota, S.; Meselhy, M. R.; Ciotti, M.; Chowdhury, B.; Hartori, M.; Owens, I. S. Gastrointestinal-distributed UDP-glucuronosyltransferase 1A10, which metabolizes estrogens and nonsteroidal antiflammatory drugs, depends upon phosphorylation. *J. Biol. Chem.* **2004**, *279*, 28320–28329.
(36) Pai, T. G.; Suiko, M.; Sakakibara, Y.; Liu, M.-C. Sulfation of flavonoids and other phenolic dietary compounds by the human cytosolic sulfotransferases. *Biochem. Biophys. Res. Commun.* **2001**, *285*, 1175–1179.
(37) Eaton, E. A.; Walle, U. K.; Lewis, A. J.; Hudson, T.; Wilson, A. A.; Walle, T. Flavonoids, potent inhibitors of the human P-form phenolsulfotransferase. Potencial role in drug metabolism and chemoprevention. *Drug Metab. Dispos.* **1996**, *24*, 232–237.
(38) Saruwatari, A.; Okamura, S.; Nakajima, Y.; Narukava, Y.; Takeda, T.; Tamura, H. Pomegranate juice inhibits sulfoconjugation in Caco-2 human colon carcinoma cells. *J. Med. Food* **2008**, *11*, 623–628.

(39) Larrosa, M.; González-Sarrías, A.; García-Conesa, M. T.; Tomás-Barberán, F. A.; Espín, J. C. Urolithins, ellagic acid-derived metabolites produced by human colonic microflora, exhibit estrogenic and antiestrogenic activities. *J. Agric. Food Chem.* **2006**, *54*, 1611–1620.

(40) Nishihashi, H.; Kanno, Y.; Tomuro, K.; Nakahama, T.; Inouye, Y. Primary structure and organ-specific expression of the rat aryl hydrocarbon receptor repressor gene. *Biol. Pharm. Bull.* **2006**, *29*, 640–647.

(41) Kim, H.; Yoon, Y. J.; Shon, J. H.; Chan, I. J.; Shin, J. G.; Liu, K. H. Inhibitory effects of fruit juices on CYP3A activity. *Drug Metab. Dispos.* **2006**, *34*, 521–523.

(42) Farkas, D.; Oleson, L. E.; Zhao, Y. L.; Harmatz, J. S.; Zinny, M. A.; Court, M. H.; Greenblatt, D. J. Pomegranate juice does not impair clearance of oral or intravenous midazolam, a probe for cytochrome P450−3A activity: Comparison with grapefruit juice. *J. Clin. Pharm.* **2007**, *47*, 286–294.

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