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Serotonin glucuronidation by Ah receptor- and oxidative stress-inducible human UDP-glucuronosyltransferase (UGT) 1A6 in Caco-2 cells

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Abstract

Caco-2 cells are a widely used model in drug development to study intestinal drug transport and metabolism. Recently, serotonin (5-hydroxytryptamine, 5-HT) has been characterized as a highly selective substrate of human UDP-glucuronosyltransferase UGT1A6 [Krishnaswamy S, Duan SX, von Moltke LL, Greenblatt DJ, Court MH. Validation of serotonin (5-hydroxytryptamine) as an in vitro substrate probe for human UDP-glucuronosyltransferase (UGT) 1A6. Drug Metab Disp 2003; 31:133–9], an isoform which conjugates planar phenols and is inducible by Ah receptor agonists and by oxidative/electrophile stress. To gain more insight into intestinal 5-HT disposition, uptake and metabolism of this neurotransmitter was studied in Caco-2 cell monolayers. It was found that 5-HT was taken up from the basolateral and to a lesser extent from the apical surface. It was mainly excreted basolaterally as 5-HT glucuronide. 5-HT UGT activity and UGT1A6 mRNA were induced by Ah receptor agonists and by oxidative stress generated by *tert*-butylhydroquinone and by isomeric thymoquinone, a potential antitumor agent and constituent of Nigella sativa seeds, commonly used as a condiment in the Middle East. While UGT1A6 induction was clearly detectable in NAD(P)H:quinone oxidoreductase 1 (NQO1)-deficient Caco-2 cells, it was not induced in NQO1-efficient HT-29 colon adenocarcinoma cells. The results suggest that – in addition to its detoxification function – intestinal UGT1A6 contributes to intestinal homeostasis of 5-HT from dietary sources and from release by enterochromaffin cells.

Keywords: UDP-glucuronosyltransferase UGT1A6; 5-Hydroxytryptamine; Serotonin; Oxidative stress; *Tert*-butylhydroquinone; Thymoquinone

1. Introduction

Glucuronidation, catalyzed by a gene superfamily of UGTs [1], represents a major phase II reaction of drug metabolism. UGTs are expressed in a tissue-specific manner and conjugate a great number of xeno- and endobiotics, the latter including steroids, bile acids, retinoids, thyroxine, fatty acids and neurotransmitters [2]. These enzymes show distinct but overlapping substrate specificity, and most substrates are glucuronidated by more than one isoform. In search for selective probe drugs of human UGTs it was recently discovered that 5-HT is a highly selective substrate of human UGT1A6 [3], which is expressed in multiple tissues, including liver, kidney, intestine and brain [4,5]. In liver of the domestic cat, a species lacking expression of functional UGT1A6 [6], 5-HT glucuronidation could not be detected [7].

5-HT is known as an important neurotransmitter in the brain. It is also stored in blood platelets and in the gastrointestinal tract, the latter representing the largest store (ca. 95%) of 5-HT in the body [8]. Most of the gastrointestinal 5-HT is present in enterochromaffin cells where it is synthesized from L-tryptophan and stored in secretory granules. From the latter it is released in response to chemical and mechanical stimuli, thereby initiating, e.g., the peristaltic reflex by acting on intrinsic sensory neurons

Abbreviations: UGT, UDP-glucuronosyltransferase; 5-HT, 5-hydroxytryptamine; 5-HT-GA, 5-hydroxytryptamine glucuronide; MUF, 4-methylumbelliferone; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; BNF, β-naphthoflavone; *t*BHQ, *tert*-butylhydroquinone; TQ, thymoquinone; NQO1, NAD(P)H:quinone oxidoreductase 1; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium

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and musculature [9]. In addition, 5-HT affects intestinal electrolyte transport in mucosal epithelial cells. To better understand serotonin homeostasis in the intestine, serotonin glucuronidation was studied in Caco-2 cell monolayers as a model of the human intestinal epithelium. 5-HT has been shown to be taken up by Caco-2 cells through the neuronal 5-HT transporter [10], where it is believed to be mainly catabolized by monoamine oxidase (MAO), and conjugated by UGTs and SULTs (sulfotransferases) [11]. Glucuronidation represents a major metabolic pathway in mice, in particular when MAO is inhibited [12].

Human Caco-2 cells which form enterocyte-like monolayers, are widely used in studies on intestinal drug transport and metabolism [13–16]. Moreover, Caco-2 cells express UGT1A6 which is inducible by Ah receptor agonists, such as TCDD and BNF, and by oxidative/electrophile stress generated by the antioxidant *t*BHQ [16,17]. Caco-2 cells are known to be NQO1-deficient and particularly sensitive to quinone cytotoxicity [18,19]. In the present study, this cell line was used (i) to study uptake and metabolism of serotonin, and (ii) to compare induction of serotonin UGT activity and of UGT1A6 mRNA by Ah receptor agonists (TCDD, BNF) and by oxidative stress mediated by *t*BHQ and the isomeric thymoquinone, a potential antitumor agent and main constituent of the volatile oil of Nigella sativa seeds, commonly used as a condiment in the Middle East [20]. The results suggest that – in addition to its role in detoxification of planar phenolic compounds – UGT1A6 may contribute to intestinal homeostasis of serotonin from dietary sources and enterochromaffin cells.

2. Materials and methods

2.1. Chemicals

Thymoquinone (2-isopropyl-5-methyl-1,4-benzoquinone), *t*BHQ and β-glucuronidase were purchased from Sigma.

2.2. Cell culture and treatment

Caco-2 cells, the high passage TC-7 clone (passage 198) [14] and HT-29 cells [21] were obtained from A. Zweibaum (Institut National de la Sante et de la Recherche Medicale, Villejuif, France) and from T. Lesuffleur (Institut Biomedical des Cordelier, Paris, France), respectively. Both cell lines were grown on 100 mm × 20 mm Falcon dishes (Becton Dickinson) in Dulbecco's modified Eagle's medium supplemented with 20% fetal bovine serum (heat-inactivated at 56° for 30 min), 25 mM glucose and 1% non-essential amino acids (Invitrogen), as described previously [16]. The medium was changed daily. Preconfluent Caco-2/TC-7 and HT29 cells were treated with 10 nM TCDD, 50 μM BNF, 80 μM *t*BHQ or 40 μM thymoquinone, dissolved in DMSO. Solvent controls contained 0.1% DMSO. Exposure time was 24 h for RT-PCR analysis and 72 h for determination of enzyme activities. For studies with monolayer cultures, cells were seeded on 12 mm i.d. Transwell polycarbonate inserts (Corning Costar Corp.) at a density of 2×10^5 cells per insert. Cells were used at day 20 in culture when the monolayer was complete, i.e., when transepithelial electrical resistance exceeded 300 Ω cm^2.

2.3. HPLC analysis of 5-HT and 5-HT-GA

HPLC was carried out with modifications as described [22]. In brief, samples were deproteinized by adding an equal volume of acetonitrile. One hundred microliters of the supernatant were dried down and reconstituted with 400 μL water. Twenty microliters thereof were injected into a HPLC system comprising a Nova-Pak C18 column. The mobile phase consisted of 20 mM $HClO_4$, pH 2.6, in 5% methanol (solvent A) and 100% methanol (solvent B). Run condition consisted of 0% solvent B for the first 10 min and a linear gradient to 20% solvent B over the next 5 min. 5-HT and 5-HT-GA were detected by fluorescence (excitation 225 nm, emission 330 nm), eluting at retention times of 7 and 10 min, respectively. 5-HT-GA was identified by its sensitivity to β-glucuronidase, and calibrated by complete conversion to 5-HT.

2.4. UGT assays

Caco-2 cells (7–10 mg protein) were homogenized by sonication in 500 μL 0.25 M sucrose containing 10 mM Tris–HCl (pH 7.4) and homogenates were stored at −80°. 5-HT UGT, MUF UGT activities and EROD (7-ethoxyresorufin *O*-deethylase) were determined as described [16]. 5-HT-GA was isolated by HPLC and quantified as described under Section 2.3. Protein was determined according to Lowry et al., using bovine serum albumin as protein standard [23].

2.5. Real-time quantitative RT-PCR analysis

Total RNA was isolated from Caco-2 cells with a phenol/chloroform extraction method using TRIzol (Invitrogen). It was reverse transcribed in 20 μL of 1× AmpliTaq PCR-Buffer II (Applied Biosystems) as follows: 500 ng of denaturated (5 min at 65°) RNA were incubated with 1 mM dNTPs, 250 ng oligo$(dT)_{15}$ (Roche), 250 ng Random Hexamer primers (Roche), 12.5 U of RNAsin RNase inhibitor (Promega), and 12.5 U of AMV Reverse Transcriptase (PeqLab) at 42° for 60 min. The samples were then heated for 5 min at 95° to terminate RT.

PCR was carried out on the Lightcycler instrument using the FastStart DNA Master SYBR Green I kit (Roche). PCR reactions contained 50 ng reverse-transcribed total RNA as

template, 3 mM $MgCl_2$, and 0.5 μM of both forward and reverse primers. Sequences of primers used were: UGT1A6 (NCBI RefSeq accession number: NM_001072): forward 5′-ATG AGA TTG TAG TGG TGG TGC-3′, reverse 5′-CCT TAA AGA AGT TCA GGG TGT-3′; UGT1A1 (NM_000463): forward 5′-AAG GGA GGA TGT GAA AGA GT-3′, reverse 5′-CAC GTA GGA GAA TGG GTT GG-3′; CYP1A1 (NM_009992): forward 5′-GTC TTT CTC TTC CTG GCT ATC-3′, reverse 5′-TAC CTG TTG TCT CTG GAG GGT-3′; cyclophilin B (NM_000942): forward 5′-GGA GAG AAA GGA TTT GGC TAC-3′, reverse 5′-ACA TGC TTG CCA TCT AGC C-3′. The following LightCycler protocols were used: denaturation step (95° for 10 min) as well as an amplification and quantification program repeated 45 times for CYP1A1 (95° for 5 s; 58° for 5 s; 72° for 14 s with a single fluorescence acquisition point), for UGT1A1 (95° for 5 s; 60° for 5 s; 72° for 15 s; to improve SYBR Green I quantification a fourth segment with a high-temperature fluorescence acquisition point at 83° for 5 s was included to the amplification cycle program), and for UGT1A6 (95° for 10 s; 60° for 5 s; 72° for 12 s with a single fluorescence acquisition point), melting curve program (60–95° with a heating rate of 0.1°/s and a continuous fluorescence acquisition). Cyclophilin B was examined as internal standard in all samples with the following settings (95° for 10 s; 68°decreasing to 58° by 0.5°/cycle, starting at the first cycle for 10 s; 72° for 16 s with a single fluorescence acquisition point) as described above.

For all quantitative assays an external calibration curve was used, based on a single-stranded DNA (ssDNA) molecule calculation. CYP1A1, UGT1A1, UGT1A6 and cyclophilin B RT-PCR products from Caco-2 cells were cloned separately in pCR2.1 using a TA cloning kit (Invitrogen). Plasmids were linearized by a unique restriction digest and serial dilutions (containing pRS2 RNA to account for the RNA content of RT samples) of each plasmid preparation from 10^2 up to 10^{10} ssDNA molecules per capillary were used in calibration curves.

2.6. Cytotoxicity assay

Cytotoxicity was tested with the MTT reduction assay using the Cell Proliferation Kit (Roche). In brief, cells were cultured for 24 h in 96-well plates (2.5×10^4 cells in 100 μL medium per well) and then exposed to *t*BHQ or thymoquinone (0–200 μM) for 24 h. MTT (10 μL) was added to each well. After 4 h incubation the medium was removed, 100 μL solubilization solution was added to each well, and the plate was incubated overnight. The formazan product was measured at 550 nm with a reference wavelength >650 nm.

2.7. Statistical analysis

Statistics was performed using Student's *t*-test.

3. Results

3.1. 5-HT uptake and excretion of 5-HT glucuronide by Caco-2 cell monolayers

5-HT was taken up both from the apical and basolateral surfaces of Caco-2 cell monolayers. Basolateral loading: The lower chamber (1.5 mL) was loaded with 150 μM 5-HT; hence, 225 nmol 5-HT were added. Within 24 h, 100 nmol (44%) were taken up by the monolayer (Fig. 1A; left panel). During this time 5-HT-GA (35%) was mainly secreted from the basolateral surface, suggesting that conjugation with glucuronic acid represents a major metabolic pathway in Caco-2 cells. Apical loading: As shown in Fig. 1A (right panel) the upper chamber (0.5 mL) contained 75 nmol 5-HT. During 24 h, the 5-HT concentration decreased in the upper chamber by 25 nmol, while only 2–3 nmol 5-HT-GA were excreted, mainly from the basolateral surfaces. The reason for the low 5-HT-GA excretion upon apical loading is unknown, but may be due to fewer neuronal serotonin transporters and/or poor absorption by the brush border. Taken together, 5-HT-GA was mostly excreted from the basolateral surface

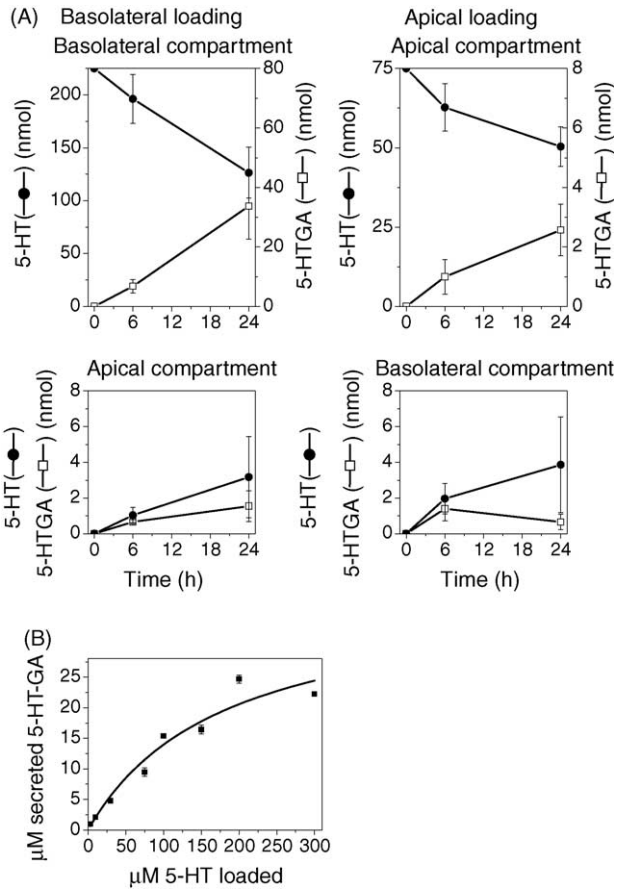

Fig. 1. (A) Uptake of 5-HT and secretion of 5-HT glucuronide from apical and basolateral Caco-2 cell surfaces after loading with 150 μM 5-HT. Data represent mean ± S.D. of four experiments. (B) Concentration dependency of 5-HT-GA formation and excretion. Three experiments after basolateral loading are listed.

presumably through the conjugate transporter MRP3, similar to previous observations with 4-methylumbelliferone glucuronide [16]. In contrast, chrysin glucuronide was mainly secreted by the apical conjugate transporter MRP2 [15]. MRP1 and MRP5 are poorly expressed in Caco-2 cells [24]. As shown in Fig. 1B, exposure of Caco-2 cell monolayers with 150 μM 5-HT was approximately saturating basolateral 5-HT-GA excretion.

3.2. Xenobiotic induction of 5-HT UGT activity and of UGT1A6 mRNA

5-HT and 4-methylumbelliferone UGT activities were clearly induced by potent Ah receptor agonists (TCDD, BNF) and by *t*BHQ-mediated oxidative stress, while CYP1A1 activity was preferentially induced by TCDD (Fig. 2A). In line with the enzyme activities, quantitative RT-PCR revealed that UGT1A6 mRNA was induced by Ah receptor agonists and oxidative stress (Fig. 2B). UGT1A1 appears to be poorly expressed in Caco-2 cells. Similar to UGT1A6, UGT1A1 has been shown to be induced by TCDD [25], but does not catalyze 5-HT glucuronidation [3]. As expected, induction of CYP1A1 mRNA was preferentially enhanced by TCDD (ca. 1000-fold), and by *t*BHQ (ca. 10-fold).

3.3. Oxidative stress-mediated UGT induction by thymoquinone and tBHQ

Thymoquinone (TQ) and *t*BHQ are presumed to undergo quinone–quinol redox cycles, thereby generating oxidative/electrophile stress. As shown in Fig. 3, thymoquinone induced 4-methylumbelliferone UGT activity similar to *t*BHQ. However, induction of gene transcription by thymoquinone was impaired by its cytotoxicity at high concentrations. Therefore thymoquinone was studied only up to 40 μM. Cytotoxicity after 24 h exposure to 80 μM thymoquinone was evident morphologically by cell vacuolization and by the MTT reduction assay. In the latter assay viability was 80% at 160 μM and 40% at 200 μM (not

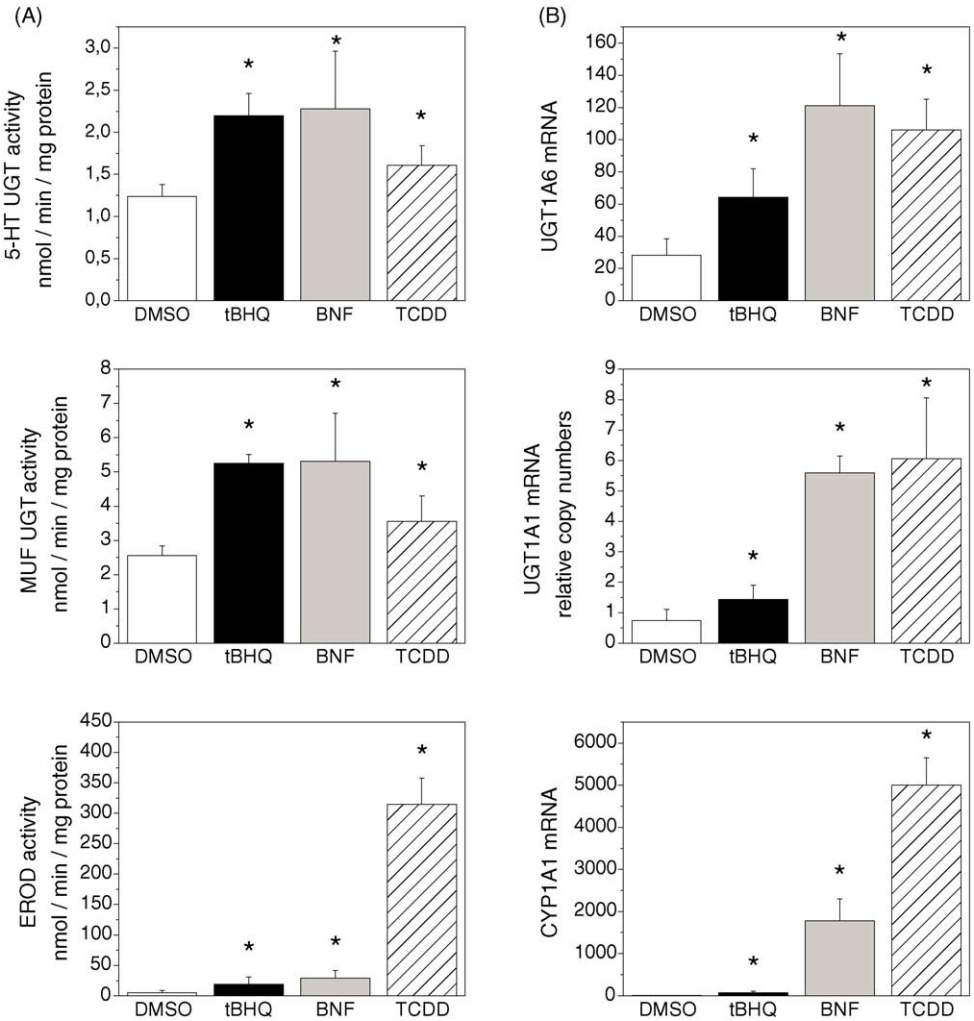

Fig. 2. (A) *t*BHQ induction of 5-HT and 4-methylumbelliferone UGT activities in comparison with CYP1A1 activity (ethoxyresorufin *O*-deethylase). Preconfluent cells were treated with the inducers indicated, and enzyme activities were determined in cell homogenates. (B) Induction of UGT1A6, UGT1A1 and CYP1A1 mRNA by TCDD, BNF and *t*BHQ. Quantitative RT-PCR was carried out as described in Section 2. Results are given as relative copy numbers, based on 1000 copies cyclophilin B. Mean ± S.D. of four experiments are listed. $^{*}P < 0.05$.

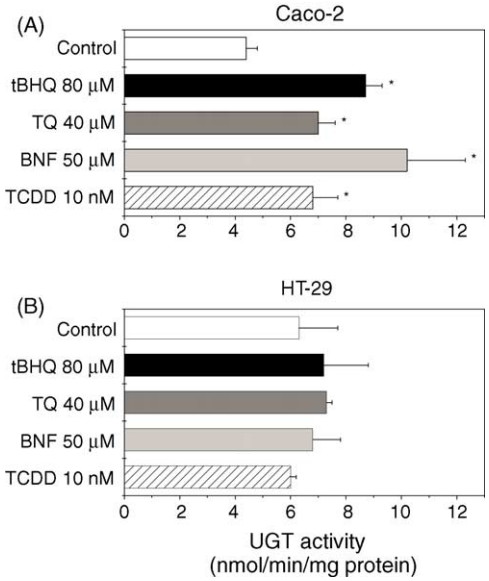

Fig. 3. (A) Induction of 4-methylumbelliferone UGT activity by AhR agonists, and by *t*BHQ- and thymoquinone (TQ)-mediated oxidative stress in NQO1-deficient Caco-2 cells. (B) Lack of induction in NQO1-efficient HT29 cells. Mean ± S.D. of four experiments are shown. $^{*}P < 0.05$.

shown). However, in studies with *t*BHQ no cytotoxicity was detectable up to 200 μM. Quinone toxicity and oxidative stress is probably due to lack of NQO1 activity in Caco-2 cells [18,19]. In contrast, in NQO1-efficient HT-29 cells thymoquinone-mediated cytotoxicity and induction of UGT activity by the above inducers was not observed, suggesting that UGT induction depends upon the cellular context. Under these conditions CYP1A1 activity was induced 49-fold by TCDD HT-29 cells (not shown), while induction was ca. 61-fold in Caco-2 cells.

4. Discussion

Caco-2 cell monolayers represent a valuable model for the intestinal epithelium. Our results demonstrate that 5-HT is absorbed by Caco-2 cell monolayers mainly from the basolateral surface, presumably by the neuronal serotonin transporter [10,26]. Reduced uptake from the apical surface remains unexplained and needs to be further explored. 5-HT is catabolized to 5-HT-GA as a major metabolite, and the glucuronide is excreted mainly from the basolateral surface, presumably via MRP3. In the intestine 5-HT is released from enterochromaffin cells during passage of the intestinal contents, thereby triggering the complex intestinal peristaltic reflex by activation of 5-HT_4 receptor leading to ascending contraction and descending relaxation [9]. 5-HT is known to be catabolized by monoamine oxidase and by conjugation with glucuronic acid and sulfate. Early studies in mice showed that urinary excretion of 5-HT as 5-HT-GA (30%) was increased to ca. 70% when monoamine oxidase was inhibited by iproniazid [12]. Taken together, our findings on 5-HT glucuronidation in Caco-2 cells suggest that UGT1A6 contributes to the homeostatic control of 5-HT when this neurotransmitter is excessively released from enterochromaffin cells. It is understood that metabolic control has to be distinguished from rapid local control of 5-HT at the 5-HT_4 receptor which is probably achieved by the serotonin uptake transporter [9,10,26].

Experiments with Caco-2 cells have shown previously that UGT1A6 is moderately inducible by both Ah receptor agonists, and by *t*BHQ-mediated oxidative stress [16,17]. In line with these findings, 5-HT UGT activity and UGT1A6 mRNA were induced by the above inducers. Homeostatic control of serotonin catabolism by UGT1A6, which – in addition to the intestine – may also be relevant in brain [4,5], has to be judged in conjunction with other major metabolic pathways, such as monoamine oxidase (MAO) and sulfotransferases. Interestingly, 5-hydroxytryptophol, one of the products of MAO together with aldehyde reductase, has recently been shown to be also a good substrate of UGT1A6 [27]. Formation of 5-hydroxytryptophol is markedly increased after alcohol consumption [28]. The role of adaptive changes of the multiple compensatory pathways of serotonin catabolism cannot be judged at present.

UGT induction by *t*BHQ was compared to oxidative stress generated by the isomeric thymoquinone, a dietary constituent of Nigella sativa seeds which are widely used as condiment in the Middle East [20]. The two compounds clearly induced UGT activity in Caco-2 cells. They are believed to generate oxidative/electrophile stress through quinone–quinol redox cycles. In studies with *t*BHQ evidence was obtained that semiquinone radicals rather than reactive oxygen species are triggering oxidative/electrophile stress [29]. Caco-2 cells are known to be particularly sensitive to quinone-mediated toxicity since they lack NQO1 activity [18,19]. The cell line was obtained from a colon adenocarcinoma of a patient who was a homozygous carrier of the NQO1*2 polymorphism, affecting 4% of the Caucasian and >20% of the Chinese population [30]. The mutant is a C-to-T base pair substitution at position 609 of the NQO1 cDNA, which codes for a proline-to-serine change at position 187 in the amino acid sequence of the protein; this mutant leads to rapid degradation of the NQO1 protein [31]. Hence, lack of NQO1 activity may be responsible for generation of oxidative/electrophile stress by *t*BHQ and thymoquinone in Caco-2 cells. When similar studies were carried out with NQO1-efficient HT-29 colon adenocarcinoma cells, no induction of UGT activity was detectable. These findings substantiate the notion that UGT1A6 induction depends upon the cellular context.

Taken together, our findings in the Caco-2 cell model suggest that – in addition to its detoxification function – Ah receptor- and oxidative stress-inducible UGT1A6 is a major contributor to intestinal homeostasis of 5-HT absorbed from dietary sources or released from entero-

chromaffin cells. 5-HT homeostasis is important in the physiology of the intestine and may contribute to side effects of drugs, such as nausea and diarrhoea.

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