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Expression of UDP-glucuronosyltransferase 1A6 isoform in Caco-2 cells stimulated with lipopolysaccharide

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Glucuronidation is an important metabolic process of detoxification in all vertebrates. The reaction is catalyzed by a multigene family of UDP-glucuronosyltransferases (UGTs) able to convert many xenobiotics and endobiotics (hydrophobic substances) to inactive, water-soluble glucuronides. The UGTs play a protective role, facilitating the elimination of potentially toxic metabolites via urine, bile and feces; therefore, impairment of UGTs may have important toxicological consequences. The regulation of UGTs during bacterial infection or inflammation is not well described. In this study, we investigated the *in vitro* effect of lipopolysaccharide (LPS) on the expression of the UGT1A6 isoform in human colon carcinoma Caco-2 cells. Results demonstrated a significant down-regulation of UGT1A6 expression, both in terms of mRNA and protein levels, and a reduced UGT activity after LPS exposure of cell cultures, suggesting a role for endotoxins on UGT regulation mechanisms.

Keywords: Caco-2 cells, lipopolysaccharide, UDP-glucuronosyltransferase, UGT1A6, mRNA

INTRODUCTION

Glucuronidation represents one of the major pathways for drug metabolism. The reaction is catalyzed by a multigene family of membrane-bound isoenzymes, UDP-glucuronosyltransferases (UGTs), and consists in the transfer of a glucuronic acid residue from UDP-glucuronic acid (UDPGA) to countless structurally unrelated substances possessing hydroxyl, amino, carboxyl or sulfhydryl groups.[1–3] This process is able to transform lipophilic substrates into hydrophilic glucuronides thus facilitating their elimination through bile, urine and feces.

Glucuronidation mainly occurs in the endoplasmic reticulum of liver and, to a lesser extent, in other extrahepatic tissues, such as kidney, skin, brain and small intestine.[4] The UGT enzymes are members of a superfamily comprising four families in mammals, but only family 1 (UGT1) and family 2 (UGT2) use UDP glucuronic acid as sugar donor.[5]

The enzymes of the UGT1A family glucuronidate several endogenous compounds such as bilirubin and steroid hormones. In particular, UGT1A6 and UGT1A7 isoforms are able to glucuronidate numerous xenobiotics.[6] Evidence for marked tissue-specific differences of human UGT expression has been reported.[7]

Investigation of UGT1A expression has focused primarily on the liver, which is considered to be the most important location of human glucuronidation,[8] but a tissue–specific expression of the UGT1A gene locus has been well characterized and has been suggested to define tissue-specific glucuronidation activity in the human digestive system.[9] An analysis of liver tissue, in fact, led to the characterization of UGT1A1, UGT1A3, UGT1A4, UGT1A6 and UGT1A9 cDNAs, while studies examining the human extrahepatic gastrointestinal tract have led to the identification of three extrahepatic UGT1A transcripts: UGT1A7, in stomach and esophagus, UGT1A8, in colon and esophagus and UGT1A10 in gastric, esophageal, biliary and colonic tissue.[10,11]

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In human liver and in intestinal biopsies, UGT1A6 expression was shown to be constitutively expressed with high interindividual variability of UGT1A6 mRNA levels.[12] Human Caco-2 cells express UGT1A6, an isoform which conjugates planar phenol, and is inducible by Ah receptor agonists and by oxidative/electrophile stress generated by the antioxidant TBHQ.[13,14]

Regulation of the human UGT1A6 gene has been studied in Caco-2 cells. Expression of UGT1A6 was found to be low and inducible by polycyclic aromatic hydrocarbons in Caco-2 cells, in contrast to high levels of constitutive expression in the A549 lung cell line.[12]

The location of the UGT1A6 transcription start site in Caco-2 cells indicated that an upstream TATA box is likely to be important for its inducible expression. In contrast, a TATA box approximately 150 bp downstream was suggested to be more important for the constitutive expression of UGT1A6 in other cell lines.[15]

Lipopolysaccharide (LPS), an ubiquitous endotoxin mediator of Gram-negative bacteria, is one of the abundant products of the intestinal normal flora.[16] It is known that intestinal epithelial cells are able to respond to LPS stimulation, even if factors regulating cell responsiveness are yet incomplete. The influence of the glucuronidation reaction by environmental contaminants is an important aspect of UGT regulation studies. The literature on UGT regulation in the intestinal epithelial cells by naturally occurring toxic substances, such as bacteria and/or their products like endotoxins, is limited. Therefore, the aim of this study was to investigate the effect of LPS treatment on expression of the UGT1A6 isoform in Caco-2 intestinal cells.

Materials and Methods

Cell culture and treatment

The Caco-2 cell line (ICLC HTL 97023-Interlab Cell Line Collection, Genova, Italy) was grown in MEM medium supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin, 100 μg/mL streptomycin, L-glutamine (2 mM), 1% non-essential amino acids (NEEA), referred to as complete medium (Life Technologies-Invitrogen, Milan, Italy).[17]

The cultures were maintained at 37°C in a humidified atmosphere containing 5% CO_2 and expanded in tissue culture flasks (75-cm^2) changing the medium daily.

For the experiments, cells (70% confluence) were treated with 80 μM *t*-butylhydroquinone (TBHQ; Sigma-Aldrich, Milan, Italy) in the presence or absence of *Salmonella enterica* sv. Typhimurium LPS (Sigma). Preliminary experiments were performed to establish the optimal dose (300 ng/mL) of LPS and the time of exposure to LPS (48 h). In another set of experiments, cells were treated as indicated above for 48 h with or without 1-h pre-treatment with the NO synthase inhibitor, 1400W (500 μM; Alexis, Milan, Italy). Untreated cells were used as control.

RNA extraction

At the end of incubation, total cellular RNA was extracted from treated cells in tissue flasks as indicated above, by the RNAzolTMbee isolation reagent (Tel-Test, Inc., Friendswood, TX, USA), according to the manufacturer's instructions. The RNA extracted was stored at −80°C until use.

Reverse transcription-polymerase chain reaction (RT-PCR) of human UGT1A6

Expression of the UGT1A6 gene was analysed by RT-PCR. Briefly, total cellular RNA extracted from Caco-2 cells was submitted to reverse transcription to obtain the cDNA, using 3 μg of total RNA, 40 U of RNase Out (a recombinant ribonuclease inhibitor; Life Technologies, Milan, Italy), 40 mU of oligo-dT with 0.5 mM dNTP (PCR Nucleotide Mix; Roche Diagnostics, Mannheim, Germany), and 40 U of Moloney murine leukemia virus reverse transcriptase (Roche Diagnostics). The reaction tubes were incubated at 37°C for 60 min, then at 95°C for 5 min and at 4°C to stop the reaction. The obtained cDNA was amplified by a Perkin Elmer 2400 thermal cycler. The hot start modification was used according to Münzel *et al*.[12] by heating the master mix containing the cDNA samples to 94°C for 1 min; FastStart Taq DNA polymerase (Roche Diagnostics) was heated to 90°C separately and added to samples. Then, samples were submitted to 30 cycles of amplification under the following conditions: 94°C for 1 min, annealing performed at 65°C for 40 s and extension at 72°C for 2 min. Thereafter, a 10-min elongation step at 72°C was included and then samples were taken to 4°C to stop the reaction. Each tube contained, in a final volume of 50 μL, 2 μL of cDNA, 200 μM dNTP (PCR Nucleotide Mix), 4 U FastStart TaqDNA polymerase (all from Roche Diagnostics), 5 μL of $MgCl_2$ buffer stock solution, and 50 pmol of primers (Invitrogen, Paisley, UK) specific for the UGT1A6 gene (GenBank accession numbers EMBL J04093; 17) and for GADPH, used as housekeeping gene control. The primers used for amplification were: for UGT1A6 (EMBL J04093; 17) forward primer, 5'GCAGGGGTTTTCTTCTTAGC 3'; reverse primer, 5'TTCTTACAGTTGATACCTCC 3'; and for GADPH forward primer, 5'-ACCACAGTCCATGCCATCAC-3'; reverse primer, 5'-TCCACCACCCTGTTGCTGTA-3'.

The resulting products were separated with an agarose gel (1.2%) and stained with ethidium bromide. They were sequenced to verify that the correct UGT isoform was amplified.

Western blot analysis

After treatment as previously described, cells were washed twice in phosphate-buffered-saline (1 × PBS), detached with scraping in ice-cold PBS, collected and centrifuged at 600 *g* for 10 min. The supernatant was removed and the pellet incubated with lysis buffer (1% [v/v] Triton X-100, 20 mM Tris-HCl, 137 mM NaCl, 10% [v/v] glycerol, 2 mM EDTA, 1 mM phenylmethylsulfonyl fluoride [PMSF], 20 μM leupeptin hemisulfate salt, 0.2 U/mL aprotinin [all from Sigma]) for 30 min on ice and then vortexed and centrifuged at 12800 *g* for 10 min. Protein concentration in the supernatant was determined by Bradford's protein assay[18] and the lysate was subjected to SDS-PAGE. Protein samples were diluted with sample buffer (0.5 M Tris-HCl, pH 6.8, 10% [v/v] glycerol, 10% [w/v] SDS, 5% [v/v] β2-mercaptoethanol, 0.05% [w/v] bromophenol blue) and then boiled for 3 min. Protein (25 μg per lane) and prestained standards (BioRad Laboratories, Hercules, CA, USA) were loaded on 10% SDS-polyacrylamide precast gels (BioRad Laboratories). After electrophoresis, the resolved proteins were transferred to nitrocellulose membrane, using a blotting buffer (20 mM Tris/150 mM glycine, pH 8, 20% [v/v] methanol). The blotting conditions were 380 mA (constant) and 80 V for 120 min. Blots were then blocked by PBS, pH 7.2, with 0.1% (v/v) Tween 20, 5% (w/v) non-fat dried milk for 1 h and washed three times with 0.1% Tween 20-PBS (T-PBS). The UGT1A6 protein was detected using a rabbit anti-UGT1A6 antibody (diluted 1 : 500; GENTEST Corporation, BD Biosciences, USA), specific for the human isoform of the UDP-glucuronosyltransferase, with molecular masses ranging between 52–62 kDa. After 60-min incubation, membranes were washed with T-PBS and detected using the goat HRP-conjugated anti-rabbit IgG (GENTEST Corporation) for 60 min at room temperature in the dark on a shaker. Finally, after three washings with T-PBS, bands were visualized by 3,3'-diaminobenzidine tetrahydrochloride (Fast DAB, Sigma). The β-actin protein level was used as a protein loading control in Western blotting.

Densitometric analysis

The bands obtained after RT-PCR and immunoblotting were submitted to densitometric analysis using ID Image Analysis Software (Kodak Digital Science). GADPH and β-actin were, respectively, used for normalisation of RT-PCR and immunoblotting products. Results were expressed as arbitrary units.

Enzyme assay

The UGT activity was measured with 0.5 mM 4-methylumbelliferone (4-MUF) as substrate, using a previously described method.[19] Briefly, Caco-2 cells (7–10 mg protein) were homogenized with 500 μL of 0.25 M sucrose containing 10 mM Tris-HCl (pH 7.4). Then, 60 μg microsomal protein was placed in a 96-well microtitre plate with pre-warmed (37°C) 0.1 mol/L Tris–HCl pH 7.3 and 20 μL 4-methylumbelliferone glucuronide (0–1000 μmol/L final concentration) such that the final volume was 0.2 mL. The reaction was started by addition of UDP-glucuronic acid (3 mM). Fluorescence was determined at 355 nm excitation and 460 nM emission in a Victor Multiplate reader (Wallac) with appearance of fluorescent substrate indicating the cleavage of the glucuronide by β-glucuronidase. Results were converted to pmol/min/mg protein using a standard curve generated with 4-MUF (0–1000 μmol/L).

Nitric oxide production

The concentration of stable nitrite, the end product of NO generation, was determined by the method described by Ding *et al.*[20] Briefly, Caco-2 cells were treated as indicated above for 48 h. At the end of treatment, culture supernatants were collected and incubated with the Griess reagent (1 : 1 v/v; Carlo Erba, Milan, Italy) for 10 min at room temperature. The absorbance of supernatants was spectrophotometrically measured at 540 nm, the NO_2^- concentration was determined by extrapolation from a $NaNO_2$ standard curve and expressed as nmol/L. To avoid interference by nitrites possibly present in the medium, in each experiment the absorbance of the unconditioned medium was assumed as the 'blank'.

Statistical analysis

Results were examined by Student's *t*-test and a P-value < 0.05 was considered statistically significant.

RESULTS

In this paper, we have investigated the effect of LPS on the expression of the UGT1A6 isoform in the Caco-2 cell line by two strategies – RT-PCR and Western blot analysis. The first approach was based on the analysis of the known sequence of the corresponding gene. Results of the RT-PCR led to the expression of a band of the

predicted size (800 bp) in Caco-2 cells submitted to different treatments (Fig. 1A). As reported by densitometric analysis of the bands obtained on the agarose gel, when TBHQ was added to Caco-2 cells, the UGT1A6 mRNA levels increased significantly ($P<0.01$) in comparison to levels obtained in TBHQ untreated cells (control; Fig. 1B). Cells treated in the presence of LPS alone exhibited mRNA levels comparable to those observed in unstimulated cells (control). Interestingly, in TBHQ pre-treated Caco-2 cells, stimulation with LPS was able to reduce significantly ($P<0.01$) the UGT1A6 mRNA levels in comparison to levels obtained in cells treated with TBHQ alone (Fig. 1A,B).

Western blot analysis revealed the expression of a protein with a molecular mass of 54 kDa, corresponding to the molecular mass of UGT1A6 in Caco-2 cells (Fig. 2A). Densitometric analysis showed that UGT1A6 expression was significantly higher ($P<0.01$) in Caco-2 cells submitted to TBHQ treatment (Fig. 2B) in comparison with untreated cells (control). Moreover, in TBHQ-treated cells, LPS was able to reduce significantly ($P=0.01$) the protein expression in comparison to cells treated with TBHQ alone (Fig. 2A,B).

The Caco-2 cells showed a basal expression of UGT1A6 activity. As expected, addition of TBHQ significantly increased ($P<0.01$) the enzyme activity (4-MUF as substrate), as shown in Figure 3. Interestingly, LPS was able to induce a significant ($P<0.01$) reduction of UGT1A6 activity in TBHQ-pretreated Caco-2 cells in comparison to TBHQ-induced cells cultured in the absence of endotoxin. No significant effects of LPS were observed in Caco-2 cells treated with LPS alone in comparison to unstimulated cells (control).

Lipopolysaccharide treatment determined a significant nitric oxide release, as shown in Figure 4. At 48 h of LPS exposure, this increase was statistically significant ($P<0.01$) in comparison to both untreated cells and TBHQ-treated cells. Nitric oxide production was ascribed to iNOS-dependent activity, since pretreatment with the specific inhibitor, 1400W, showed a significant reduction ($P<0.01$) of NO release both in LPS-stimulated and in TBHQ + LPS-treated Caco-2 cells as reported in Figure 4A.

This NO release by LPS-treated cells may be involved in UGT1A6 down-regulation. In fact, as shown in Figure 4B, in cells pretreated with the iNOS inhibitor 1400W, before TBHQ + LPS treatment, both mRNA and protein levels of UGT1A6 were comparable to those observed in cells stimulated with TBHQ alone.

DISCUSSION

We present an *in vitro* model to study the effect of LPS on intestinal UGT regulation after exposure of Caco-2

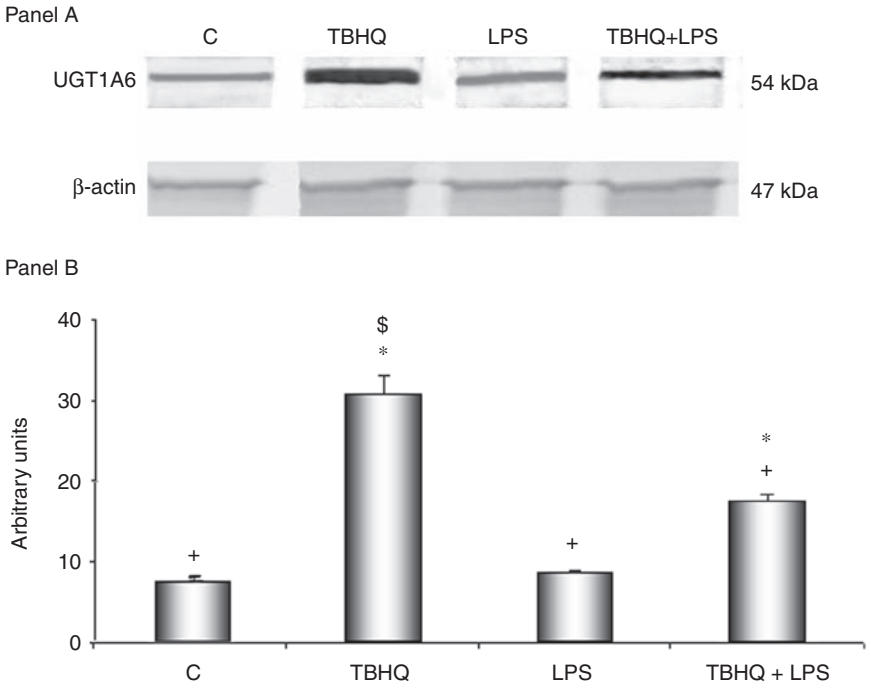

Fig. 1. Reverse transcription-PCR analysis of UGT1A6 in Caco-2 cell line. (A) Expression of UGT1A6 mRNA in cells grown in medium alone (C), treated with TBHQ (TBHQ), treated with LPS (LPS), or treated with LPS in the presence of TBHQ (TBHQ + LPS). Experiments were repeated six times. (B) Densitometric analysis of UGT1A6 mRNA expression, represented in arbitrary units, after normalisation against GADPH. Results are given by mean ± SD of six repeated experiments. $^{*}P<0.01$, significantly different vs control; $^{+}P<0.01$, significantly different vs TBHQ; $^{\$}P<0.01$, significantly different vs LPS.

cells to endotoxin. Our results provided, for the first time in an *in vitro* model of intestinal cells, evidence that LPS is able to down-regulate not only the expression of UGT1A6 both at transcriptional and post-transcriptional levels in Caco-2 cells, but also its enzymatic activity using the 4-MUF as substrate.

Caco-2 cells, established from a human colonic adenocarcinoma, form enterocyte-like monolayers and are widely used as a model for intestinal epithelium studies because of their ability to express morphological and biochemical features of adult enterocytes (microvilli on the apical surface, tight junctions and express typical

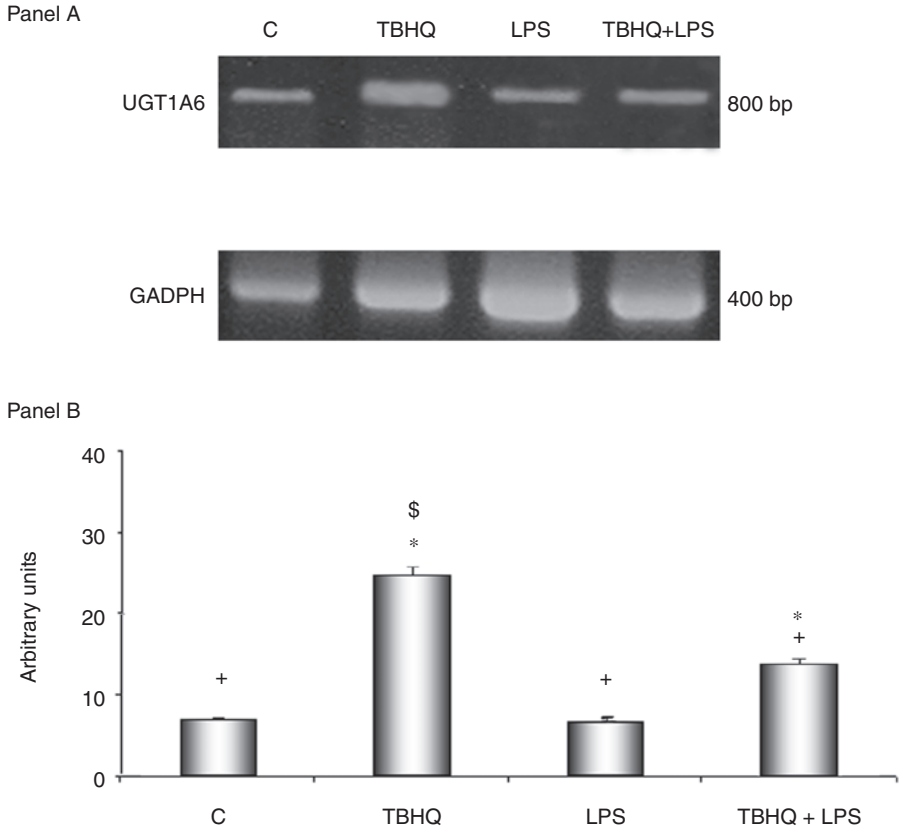

Fig. 2. Expression of UGT1A6 protein in Caco-2 cell line. (A) Immunoblot analysis of UGT1A6 in cells grown in medium alone (C), treated with TBHQ (TBHQ), treated with LPS (LPS), or treated with LPS in presence of TBHQ (TBHQ + LPS). Experiments were repeated six times. (B) Densitometric analysis of UGT1A6 protein expression, represented in arbitrary units, after normalisation against β-actin. Results are given as mean ± SD of six separated experiments. $^{*}P < 0.01$, significantly different vs control; $^{+}P < 0.01$, significantly different vs TBHQ; $^{\$}P < 0.01$, significantly different vs LPS.

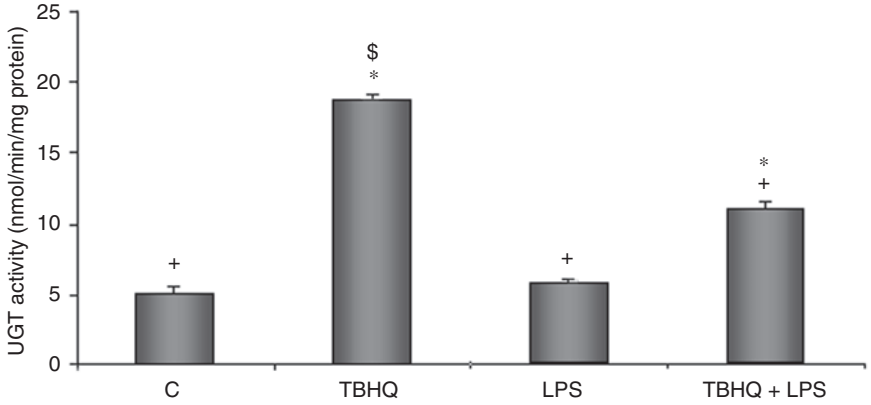

Fig. 3. Induction of UGT activity (4-MUF as substrate) in Caco-2 cells. Unstimulated cells (control, C); TBHQ-pretreated cells (TBHQ), LPS-stimulated cells (LPS); and LPS-stimulated cells in the presence of the inducer TBHQ (TBHQ + LPS). Results are given as means ± SD of six independent experiments. $^{*}P < 0.01$, significantly different vs control; $^{+}P < 0.01$, significantly different vs TBHQ; $^{\$}P < 0.01$, significantly different vs LPS.

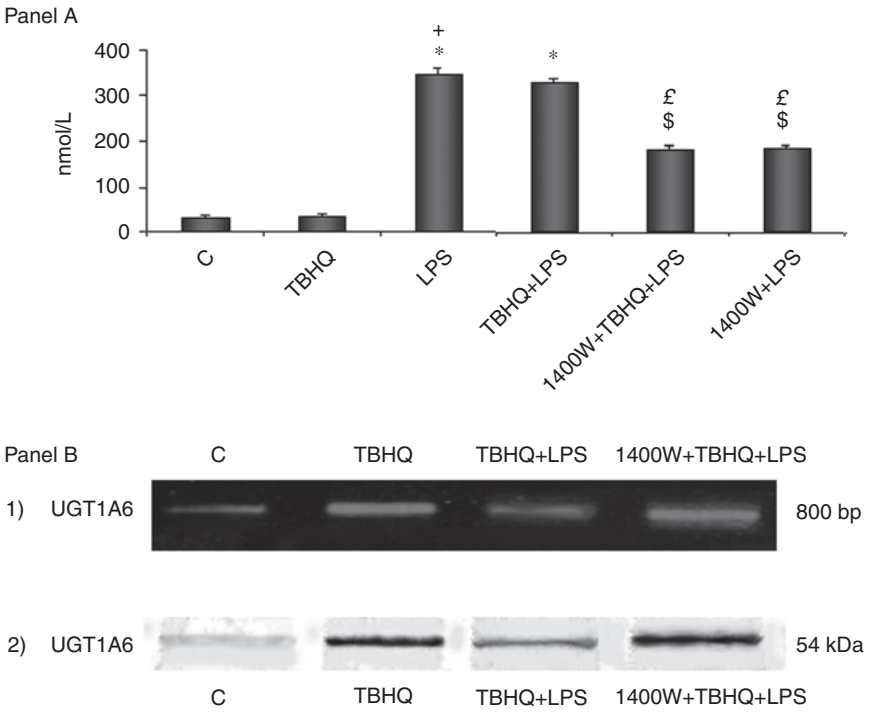

Fig. 4. Nitric oxide production in Caco-2 cells (A): unstimulated cells (control, C); TBHQ-pretreated cells (TBHQ), LPS-stimulated cells (LPS); LPS-stimulated cells in the presence of the inducer TBHQ (TBHQ + LPS); LPS-stimulated cells in the presence of the inducer TBHQ and the iNOS specific inhibitor 1400W (1400 W + TBHQ + LPS); LPS-stimulated cells in the presence of the iNOS specific inhibitor 1400W (1400 W + LPS). (B) Reverse transcription-PCR analysis of UGT1A6 mRNA (1) and immunoblot analysis of UGT1A6 protein expression (2) in Caco-2 cell line. Unstimulated cells (control, C); TBHQ-pretreated cells (TBHQ), LPS-stimulated cells in the presence of the inducer TBHQ (TBHQ + LPS); LPS-stimulated cells in the presence of the inducer TBHQ and the iNOS specific inhibitor 1400W (1400 W + TBHQ + LPS). Results are given as mean ± SD of six independent experiments. $^{*}P < 0.01$, significantly different vs control; $^{+}P < 0.01$, significantly different vs TBHQ; $^{\$}P < 0.01$, significantly different vs LPS; $^{£}P < 0.01$, significantly different vs TBHQ + LPS.

hydrolases and nutrient transporters).[21,22] One feature of this cell line is that the cells spontaneously undergo enterocytic differentiation during culture. This differentiation begins when cells form a monolayer and is completed after 20 d. Experiments with Caco-2 cells demonstrated that they express UGT1A6 constitutively and that UGT1A6 mRNA levels are very stable in these cells.[23]

There is evidence that many endogenous substances including bile acids, hormones, acyl-coenzyme A and nucleotides, inhibit or down-regulate UGT activity. The endogenous bile acids (cholic acid, chenodeoxycholic acid, and deoxycholic acid) have been reported to inhibit bilirubin glucuronidation in digitonin-disrupted rat liver microsomes.[24] Impairment of the glucuronidation reaction by clinically used drugs is an important aspect of UGT inhibition studies. It was reported that some drugs are able to interfere with the glucuronidation of endogenous component, therapeutic agents, and xenobiotics.[25]

A number of environmental contaminants (*e.g.* industrial pollutants, heavy metals, and pesticides) were reported to produce a pronounced inhibitory effect on UGT activity in different animal models.[26–29] Also, several antibiotics, carcinogenic mycostatins, tannine, safrols, present in the diet were reported to have inhibitory effects on UGT.[24,30] Finally, a number of factors regulating gene expression of UGT1A, including UGT1A6, and the subsequent glucuronidation activity in Caco-2 cells were recently reported.[31,32]

Synthetic phenolic antioxidants, such as *t*-butylhydroquinone (TBHQ), are commonly used as food preservatives due to their potent anti-lipid peroxidation activity whereas natural phenolic antioxidants exist in a wide range of edible plants and animal tissues.[33–35] Studies in rodents demonstrated that UGT1A6 is regulated by phenolic antioxidants/eletrophiles called antioxidant-type inducers.[36] Reports demonstrated that phenolic antioxidants potently inhibit signal-induced TNF-α transcription and suggest a mechanism of anti-inflammation by the antioxidants through control of cytokine or other inflammatory mediators, including prostaglandins.[37,38]

To date, very little data are available about UGT expression during LPS-induced inflammation and how endotoxin may interferes on UGT modulation. In a recent report performed on a murine model, it was demonstrated that *in vivo* LPS exposure, as well as

experimental infection with Gram-negative bacteria, are able to down-regulate or up-regulate UGT isoforms depending on the cell type.[39]

In rat astrocyte cultures, an increase in glucuronidation activity was observed after LPS treatment, associated with an up-regulation of the UGT1A6 isoform mRNA level, mediated by pro-inflammatory signals.[40]

Other studies led support to the idea that the effects on UGT during inflammation may be cytokine dependent, since IL-1α, TNF-α and IL-6 cause inhibition of glucuronidation in isolated pig hepatocytes.[41] Although we did not evaluate cytokine release by treated Caco-2 cells, current evidence indicates that LPS is able to trigger a long-term secretion of cytokines, as observed during sepsis, which in turn provides a sustained production of inflammatory mediators, including NO.[42,43] In this context, we observed that LPS was able to induce a significant release of NO in Caco-2 cells, which could be involved in UGT down-regulation since NO inhibition was able to restore the levels of UGT expression. This conclusion appears to be in accordance with previous results reporting a possible role for NO in the inhibition of glucuronidation.[41] Moreover, in order to investigate the route by which LPS exerts its effects on UGT expression, we performed experiments of competition for the binding to Toll-like receptor (TLR)-4 using a specific antibody directed against TLR-4 before LPS treatment. In this context, we did not observe any effect exerted by TLR-4 on UGT expression (data not reported). This conclusion seems to be in accordance with that observed by other authors in a murine model of kidney and liver, reporting that Gram-negative infection did not require a functional TLR-4.[39] It remains to be established if this influence by endotoxin is a UGT1A6-specific phenomenon or a general effect for all UGT isoforms and the possible mechanisms involved in this action. We are currently studying if LPS is able to exert similar or different effects on UGT expression in other cell types of the gastrointestinal tract. The intestinal epithelium cells are constantly exposed to toxic xenobiotics, including Gram-negative bacteria and their components, such as endotoxins. Therefore, the results of this work suggest that LPS down-regulation of UGT1A6 in Caco-2 cells may impair conjugation reactions in the bowel, thus interfering with a detoxification pathway of potentially toxic compounds.

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