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Effect of spironolactone on the expression of rat hepatic UDP-glucuronosyltransferase

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Abstract

Spironolactone (SL) increases the glucuronidation rate of several compounds. We analyzed the molecular basis of changes occurring in major rat liver UDP-glucuronosyltransferase (UGT) family 1 isoforms and in UGT2B1, a relevant isoform of family 2, in response to SL. UGT activity toward bilirubin, ethynylestradiol and *p*-nitrophenol was assayed in native and activated microsomes. Protein and mRNA levels were determined by Western and Northern blotting. The lipid composition and physicochemical properties of the microsomal membrane were also analyzed. Glucuronidation rates of bilirubin and ethynylestradiol (at both 3-OH and 17β-OH positions), determined in UDP-*N*-acetylglucosamine-activated membranes, were increased in SL group. Western blot analysis revealed increased levels of UGT1A1 and 1A5 (bilirubin and 3-OH ethynylestradiol conjugation), and 2B1 (17β-OH ethynylestradiol conjugation). Northern blot studies suggested transcriptional regulation by the steroid. Analysis of UGT activity in native vs. alamethicin-activated microsomes indicated increased latency, which was not associated to changes in physicochemical properties of the microsomal membrane. *p*-Nitrophenol glucuronidation rate and mRNA and protein levels of UGT1A6, the main isoform conjugating planar phenols, were not affected by the inducer. The data suggest transcriptional regulation of specific isoforms of hepatic UGT by SL, thus explaining previously reported increases in UGT activity toward selective substrates.

Keywords: UDP-glucuronosyltransferase; Spironolactone; Rat liver; *p*-Nitrophenol; Bilirubin; Ethynylestradiol

1. Introduction

UGTs are a superfamily of membrane-bound enzymes that metabolize both endogenous compounds (such as bilirubin, bile acids and steroid and thyroid hormones), and exogenous compounds (such as food additives, therapeutic drugs and environmental pollutants), thereby facilitating their excretion from the body [1,2]. Based on the nucleotide and amino acid sequences, UGT isoforms in mammals are grouped in two major families termed 1 and 2. The structure of the UGT1 gene contains several first exons encoding isoform-specific sequences that confer aglycone specificity, followed by a set of common exons (2–5) encoding the C-terminal sequence that is identical in all UGT1 isoforms [3–5]. The transcript then undergoes alternative splicing. In contrast, UGT family 2 isoforms are each derived from an individual gene and are responsible for the glucuronidation of steroids and opioids [6,7].

UGT activity is affected by many factors including aging, diet, hormones, diseases and enzyme inducers [1,8,9]. Glucuronidation is under complex regulation. The first control may occur either at the gene transcription level, resulting in changes in mRNA and protein levels, and/or at the level of post-translational processing. The second one is exerted on the modulation of the functional state (or activity per molecule) of the enzyme, because of the association of UGT with the lipid environment [10], the restriction of the cosubstrate UDP-glucuronic acid (UDPGA) to access the enzyme active site [11,12] and/or the protein–protein interactions derived from oligomer formation [13–15].

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Abbreviations: SL, spironolactone; UGT, UDP-glucuronosyltransferase; UDPGA, UDP-glucuronic acid; UDP-N-AG, UDP-*N*-acetylglucosamine.

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Different forms of UGT are selectively induced by the administration of various chemical compounds [16]. The induction of individual enzymes of UGT in rat liver has been extensively studied for classical inducers such as phenobarbital, clofibrate, 3-methylcholanthrene and dexamethasone [3,17,18], revealing transcriptional regulation. SL, a diuretic widely used in patients with edema or ascites [19], also induces the activity of several hepatic microsomal enzymes including UGT [20–22]. This could result in increased metabolism of drugs coadministered with the diuretic; e.g. drugs suffering phase I metabolism have a shorter half-life in cirrhotic patients receiving SL [23].

Characterization of SL effect on the different isoforms of UGT has been performed only in terms of activity toward the model substrates reacting with the UGT1 family isoforms. Thus, hepatic UGT activity toward bilirubin was reported to be substantially increased in rats pre-treated with SL [20,21] whereas the conjugation rate of planar phenols was not affected [22]. Because of overlapping of substrate specificity among the different isoforms and multifactorial regulation of UGT activity, previous results on enzyme activity do not necessarily reflect changes in protein and/or mRNA levels. Microsomal lipids were shown to influence UGT activity [10]. It is also known that K^+ canrenoate (an active metabolite of SL) induces an increase in the cholesterol/phospholipid molar ratio and a decrease in plasma membrane fluidity in rat liver [24]. Consequently, it is possible that these steroids affect UGT activity by modulating enzyme environment, in addition to gene expression.

In the present study we analyzed the effect of SL administration on expression of UGT family 1 isoforms (1A1, 1A5 and 1A6) and on UGT2B1, a relevant family 2 isoform, in rat liver. Protein and mRNA levels as well as activity toward the classical substrates of the different isoforms were systematically determined. The effect of SL on UGT latency and on lipid composition and physicochemical properties of the microsomal membrane was also evaluated.

2. Materials and methods

2.1. Chemicals

17α-[6,7-^{3}H-*N*]Ethynylestradiol (49.1 Ci/mmol) was from New England Nuclear. SL, bilirubin, UDPGA (ammonium salt), UDP-*N*-acetylglucosamine (UDP-N-AG), *p*-nitrophenol, mannose 6-phosphate (disodium salt), D-saccharic acid 1,4-lactone, alamethicin, 1,6-diphenyl-1,3,5-hexatriene (DPH) and non-labeled ethynylestradiol were purchased from Sigma Chemical Co. All other reagents were of the highest grade commercially available.

2.2. Animals and treatment

Adult male Wistar rats (300–360 g) were used throughout. They were maintained *ad libitum* on a standard laboratory pellet diet and were allowed free access to water and saline solution during treatment. The animals were injected with SL i.p. at a daily dose of 200 μmol/kg body wt, dissolved in propylene glycol, for 4 consecutive days, with the last dose administered 3–4 hr before the sacrifice (SL group). This dose was previously found to maximally increase bilirubin UGT activity in rat liver microsomes [25]. Control rats were injected with propylene glycol (vehicle of SL) according to the same schedule described above. All procedures were conducted in accordance with NIH guidelines for the Care and Use of Laboratory Animals.

2.3. Preparation of microsomal fractions

All animals were killed by bleeding after cardiac puncture under pentobarbital anesthesia (50 mg/kg body wt) between 11:00 and 12:00 hr, to avoid possible effects of diurnal variations. A little portion of livers were promptly removed and snap frozen in liquid nitrogen for RNA analysis. The rest of the livers were perfused *in situ* with ice-cooled 0.9% NaCl solution through the portal vein. Liver homogenates (25% (w/v)) were prepared in 0.15 M Tris–HCl buffer, pH 7.40. Microsomal fractions were obtained by ultracentrifugation at 105,000 *g* for 1 hr at 4° as previously described [26]. Protein content was determined by the biuret reaction [27], using a commercial standard (Wiener Lab).

2.4. Evaluation of microsomal membrane integrity

To establish whether integrity of microsomes is affected by SL treatment, mannose 6-phosphatase activity was determined in native and fully-activated preparations as previously described [28]. Mannose 6-phosphatase latency was calculated and expressed as percentage [29].

2.5. Enzyme assay

UGT activities were evaluated in microsomes physiologically activated with UDP-N-AG (2.0 mM final concentration). Assay conditions for the determination of glucuronidation rate of *p*-nitrophenol, bilirubin and ethynylestradiol conjugated in 3-OH and 17β-OH positions were as previously described [28,30,31]. It is noted that isoforms of the UGT1 family are involved in the conjugation of bilirubin (UGT1A1 and 1A5), *p*-nitrophenol (UGT1A6) [18] and ethynylestradiol in position 3-OH (UGT1A1), and isoforms of the UGT2 family conjugate ethynylestradiol in position 17β-OH (UGT2B1 among others) [32,33].

Since the active site of the UGTs resides in the lumen of the endoplasmic reticulum, enzyme activities are highly dependent on the status of the microsomal membrane and changes in this feature may be reflected by changes in the percent of activation by a perturbant agent referred to native enzyme activity (latency). In additional experiments,

UGT activity toward bilirubin and *p*-nitrophenol was also evaluated in native (non-activated) microsomes and in microsomes-activated with the pore-forming alamethicin. This agent neither profoundly alters the microsomal vesicular structure nor solubilizes appreciable amounts of microsomal proteins [34,35]. Because of its pore-forming action, alamethicin likely allows substrates to freely reach UGT catalytic sites. The ratio alamethicin/microsomal protein used was 50 μg/mg of protein for both bilirubin and *p*-nitrophenol UGT activity determination, which is in the range of alamethicin concentration demonstrated to fully activate *p*-nitrophenol UGT [36] and other substrates [37,38]. For activation, microsomes were pre-incubated with alamethicin for 2 min at room temperature. Enzyme assay conditions were as previously described [28,30].

2.6. Western blot analysis

Polyclonal anti-peptide antibodies that specifically recognize the 1A1, 1A5 and 1A6 isoforms belonging to UGT family 1 as well as an antibody developed against a peptide common to all isoforms of the same group (1A) [18] and a specific antibody against isoform 2B1 (UGT family 2) [14] were used in Western blot studies. Western analyses were performed as described previously [31]. Immunoreactive bands were quantified by densitometry (Shimadzu CS-9000, Shimadzu Corporation).

2.7. Northern blot analysis

UGT1A1, 1A5 and 1A6 probes consisting of 293, 308 and 317 bp cDNA fragments, respectively were prepare by RT–PCR as we have described [31]. The content of mRNA encoding the family 2 isoform UGT2B1 was analyzed using a full-length rat probe [39], which was generously provided by Dr. Peter Mackenzie (Flinders University of South Australia). A single-stranded 26-mer oligoprobe to 28S rRNA [40] was synthesized by Integrated DNA Technologies, Inc.

Total RNA was isolated from liver samples frozen in liquid nitrogen using a commercial kit (SV total RNA isolation system, PROMEGA) following the instructions provided by the manufacturer. Northern studies were performed as previously described [31]. The hybridization bands were quantified by densitometry (Shimadzu CS-9000, Shimadzu Corporation), which was done in the linear range of the film.

2.8. Lipid analysis and polarization studies

Microsomal lipids were extracted by the procedure of Folch *et al.* [41]. Total cholesterol, lipid phosphate content, phospholipid classes, fatty acid composition and steady-state fluorescence anisotropy of DPH (*r*) were determined as described previously [42].

2.9. Statistical analysis

Data were presented as means ± SD except for densitometric analysis of Northern blot studies in which only mean values were calculated. Statistical analysis was performed using Student's *t*-test. Values of $P < 0.05$ were considered to be statistically significant.

3. Results

3.1. UGT activities

First, we evaluated the functional integrity of microsomal vesicles. Hepatic microsomes from control group exhibited a latency of mannose 6-phosphatase of 89–95% (N = 7) that was not affected by SL treatment (88–94%, N = 11). Thus, changes in UGT activity determined in native or UDP-N-AG-activated microsomes in response to SL administration could not be attributed to artifacts of the isolation procedure, and any eventual change in the functional features of UGT, detected by activation analysis, should not be attributed to an altered microsomes integrity.

Fig. 1 shows enzyme activities determined in the presence of UDP-N-AG, which is considered a physiological activator of UGTs [11,12]. In addition, under this experimental condition, no substantial disturbance of the membrane environment that could affect catalytic activity of UGTs is expected. Conjugation rates of bilirubin and ethynylestradiol in position 3-OH and 17β-OH exhibited a significant increase in SL-treated rats (about 150, 100 and 120% over controls, respectively), whereas *p*-nitrophenol glucuronidation was not affected.

To evaluate if SL was able to produce changes in UGT latency, bilirubin and *p*-nitrophenol conjugation rates were also determined in native and alamethicin-activated

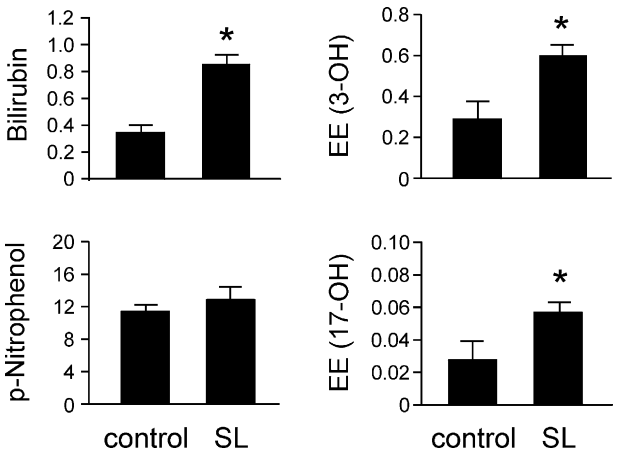

Fig. 1. Effect of SL administration on UGT activity. UGT activities toward ethynylestradiol (EE) (3-OH and 17β-OH conjugation), bilirubin and *p*-nitrophenol were determined in the presence of the physiological activator UDP-N-AG (2 mM). Conjugating activity is expressed as nmol/min per mg of protein. Data are mean values ±SD, where N = 4–8. *Significantly different from control rats ($P < 0.05$).

Table 1
Effect of alamethicin on UGT activity

	p-Nitrophenol		Bilirubin	
	Control	SL	Control	SL
Native	6.0 ± 0.8	5.8 ± 0.7	0.20 ± 0.07	0.46 ± 0.03*
Alamethicin	22.3 ± 2.8	27.4 ± 2.6	0.99 ± 0.33	3.96 ± 1.07*
Activation	272 ± 27%	373 ± 121%	394 ± 86%	759 ± 167%*

Glucuronidation rate of *p*-nitrophenol and bilirubin in native (latent) and alamethicin-activated microsomes. Conjugating activity is expressed as nmol/min per mg of protein. Results are mean values ±SD of three animals per group.

* Significantly different from controls ($P < 0.05$).

microsomes. The data shown in Table 1 indicate that SL did not affect *p*-nitrophenol conjugation rate in any assay condition. In contrast, bilirubin conjugation was significantly increased in response to SL in both native (130% over controls) and fully-activated (300% over controls) microsomes. The greater activation in SL-treated rats suggests that, despite of a major number of enzyme molecules, each one shows a higher degree of restriction to express its activity (i.e. lower activity per molecule) compared with controls.

3.2. Immunoblotting analysis

Samples from three rats per group were loaded in the same gel to perform statistical analysis. Fig. 2 shows a significant increase in the intensity of immunoreactive bands in SL-treated rats for all the isoforms tested except UGT1A6. The increases for UGT1A1, 1A5 and 2B1 were of about 450, 400 and 300% over controls, respectively. The immunoblot performed with the 1A antibody, that recognizes the most relevant UGT1 isoforms [18], confirmed the induction observed for UGT1A1 and 1A5. UGT1A band was increased by about 330% in response to SL.

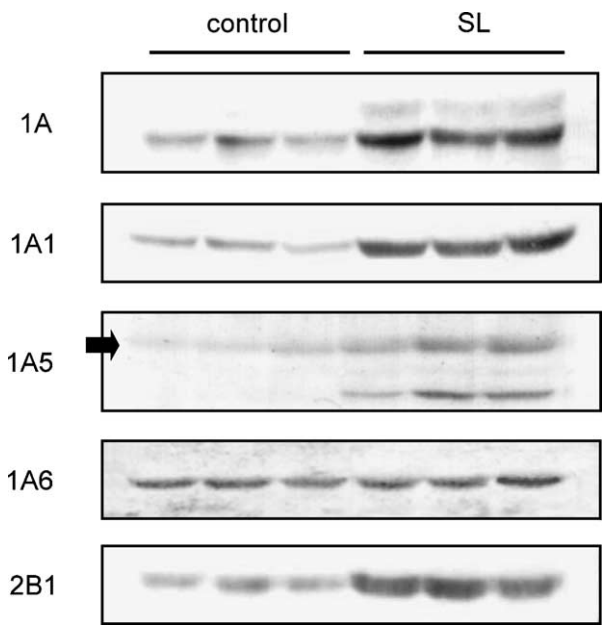

Fig. 2. Effect of SL treatment on the level of UGT isoforms. Equal amounts of microsomal protein (15 μg) were loaded in each lane (lane 1–3: control rats; lanes 4–6: SL-treated rats). The densitometric analysis in arbitrary units (means ± SD, N = 3) revealed more protein in SL-treated rats for UGT1A1 (164 ± 11 vs. 30 ± 12; $P < 0.05$), UGT1A5 (20 ± 9 vs. 4 ± 2; $P < 0.05$), and UGT2B1 (201 ± 43 vs. 50 ± 18; $P < 0.05$). The antibody recognizing a common epitope of family 1 isozymes (UGT1A) also revealed more protein in SL-treated animals (287 ± 70 arbitrary units vs. 67 ± 34 arbitrary units; $P < 0.05$). SL did not affect the level of UGT1A6 (61 ± 17 arbitrary units vs. 43 ± 9 arbitrary units).

3.3. Northern blot studies

Fig. 3 shows that treatment with SL clearly increased UGT1A1, 1A5 and 2B1 mRNA levels (about 100, 170 and 160% over controls, respectively). The data suggest transcriptional regulation of UGT by the steroid. The discrepancy in the magnitude of increase between protein and mRNA content could result from a different kinetic of increase with time. Usually mRNA levels vary with time along the period of treatment with an inducer and the peak of increase precedes protein increase. We analyzed mRNA levels 3–4 hr after the last SL injection, which might not coincide with maximal synthesis of UGT mRNA. Fig. 3 also shows that the level of mRNA encoding UGT1A6 was not affected by SL.

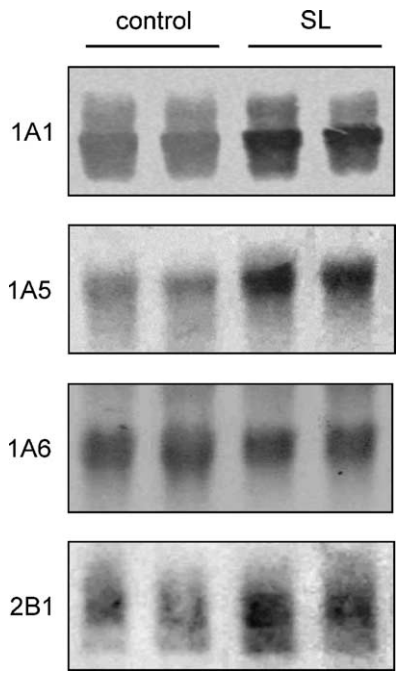

Fig. 3. Effect of SL administration on UGT mRNA levels. To correct for differences in total RNA loading and transfer among the lanes, the content of 28S rRNA was also estimated (images not shown). The densitometric analysis was performed on both specific UGT mRNAs and 28S rRNA (lanes 1 and 2: control rats; lanes 3 and 4: SL-treated rats). The data (arbitrary units of mRNAs densitometry relative to the corresponding 28S values, N = 2) revealed increased levels for UGT1A1, 1A5 and 2B1 in SL-treated rats (mean values of 287 vs. 143, 198 vs. 73 and 230 vs. 88, respectively). UGT1A6 mRNA was not affected by SL (210 arbitrary units vs. 205 arbitrary units).

Table 2
Interlipid relationships, UI of total fatty acids and membrane fluidity of microsomal membranes

	Controls	SL
Cholesterol/phospholipids ratio (mol/mol)	0.15 ± 0.01	0.14 ± 0.01
Phosphatidylcholine/phosphatidylinositol ratio (mol/mol)	15.1 ± 3.2	14.9 ± 2.8
UI	1.35 ± 0.04	1.28 ± 0.05
Fluorescence anisotropy (r)	0.116 ± 0.004	0.116 ± 0.003

The methyl esters of the fatty acids 16:0, 16:1, 16:2, 18:0, 18:1, 18:2, 18:3, 20:3, 20:4 and 22:6 were analyzed by GLC, and the UI was calculated as $\sum n_i x_i$/FA, where n_i, number of double bonds in each fatty acid, x_i, moles of each fatty acid, and FA, total moles of fatty acid. Values are means ±SD of three animals per group.

3.4. Microsomal membrane composition and membrane fluidity

UGTs are membrane-bound enzymes and the endoplasmic reticulum membrane represents a barrier for the access of UDPGA (hydrophilic co-substrate) to the active site [11,12] and for the product to be released [43]. Additionally, due to lipid–protein interactions, the physicochemical properties of the microsomal membrane may be an important factor regulating its functional state [10]. Since K^+ canrenoate (an active metabolite of SL) induces changes in the interlipid relationships and membrane fluidity in plasma membrane of rat liver [24], it was of interest to evaluate the composition of the microsomal membrane after SL treatment. Analysis of phospholipid composition demonstrated no changes in the relative content of phosphatidylcholine, phosphatidylethanolamine, sphingomyelin and phosphatidylserine + phosphatidylinositol in response to SL (data not shown). Data on cholesterol/phospholipids and phosphatidylcholine/sphingomyelin molar ratios as well as the unsaturation index (UI) for total fatty acids are shown in Table 2. None of these parameters were significantly affected by SL. Relative content of major fatty acids was neither affected by SL (data not shown). Lack of alteration in microsomal membrane lipid composition in response to SL was consistent with unaltered fluidity of the microsomes determined through fluorescence anisotropy of DPH (r) (see Table 2).

4. Discussion

UGT isoforms may be selectively induced by the administration of chemical compounds. UGT1A1 is a constitutively expressed form involved in conjugation of the endogenous substrate bilirubin and also in conjugation of the synthetic estrogen ethynylestradiol in position 3-OH [32]. It was reported that its transcription is stimulated by administration of dexamethasone or clofibrate to rats [3,18]. In the same report the authors found that UGT1A5, which belongs to bilirubin cluster [18], is similarly induced by these agents, and that UGT1A6 and 1A7, isoforms associated with p-nitrophenol conjugation, are not affected by dexamethasone or clofibrate but induced by 3-methylcholantrene. Level of mRNA encoding UGT2B1, an isoform involved in conjugation of ethynylestradiol in position 17β-OH, was increased in rat liver in response to phenobarbital [17], whereas UGT1 isozymes are not substantially affected by this agent [3,18]. The present study suggests transcriptional regulation of UGT1A1, UGT1A5 and 2B1 by SL. Neither mRNA nor protein level of the UGT1A6 isoform were affected by the steroid, suggesting an independent action on different UGT1 members. The mechanism regulating the production of UGT1 mRNAs by alternative usage of multiple promoters and first exons is complex. The specificity of the effect of SL on UGT1 family add more evidence to the idea that isoform-specific promoters exist in the 5′-flanking regions of each first exon as was suggested [3].

We detected an increase in the activity toward bilirubin and ethynylestradiol conjugation in position 3-OH, agreeing well with increases in the level of UGT1A1 and 1A5 protein, and in 17β-OH ethynylestradiol glucuronidation, agreeing well with increased level in UGT2B1 protein. Enzyme activities determined in UDP-N-AG-activated microsomes increased about 125% in average whereas protein levels showed increases of 300–450% in response to the steroid. Dissociation between changes in activity and expression of UGT may derive from the particular conditions of the enzyme activity assay. Pre-treatment of microsomal membranes with detergents or pore-forming agents are known to maximally activate UGT [36–38]. In contrast, UDP-N-AG addition to the incubation medium only partially activates UGT by increasing permeability of the microsomal membrane to the cosubstrate UDPGA [11,12]. Because of its pore-forming action, alamethicin most probably facilitates the access of the reagents (substrates) to and the release of the product from the lumen of the microsomal vesicles. Our data on the degree of activation by alamethicin, which was about 2-fold higher for microsomes isolated from SL-treated rats, suggest that the induced enzyme may not be totally active under physiological conditions. Physicochemical properties of the enzyme environment may condition enzyme activity, in addition to the natural barrier imposed by the microsomal membrane [10]. We evaluated the effect of SL on lipid composition and fluidity of the microsomal membrane. The data indicated that, in contrast to what was reported for plasma membrane [24], the steroid did not substantially affect these parameters. In consequence, the higher latency observed in SL-treated animals most probably resulted from restrictions in transport of substrates and/or product across the membrane. A possibility arises that the proteins involve in these transport processes are not induced by SL at the same degree as UGTs. Further studies are necessary to investigate this possibility.

The data on enzyme activity induction observed in fully-activated microsomes agreed well in magnitude with protein upregulation and confirmed the increased number of

catalytic units in treated rats. The use of UDP-N-AG-activated membranes was instrumental in demonstrating the effect of SL under physiological conditions of incubation and more likely mimicked the *in vivo* situation. In support to this assumption, infusion of an overload of unconjugated bilirubin to rats, leading to maximal biliary excretion of the conjugated pigment, demonstrated only a 1.6-fold increase in response to SL [21]. According to Van Steenbergen and Fevery [44] maximal biliary excretion of bilirubin depends on UGT activity determined in microsomal membranes. Similarly, hepatocytes isolated from SL-treated rats incubated with unconjugated bilirubin showed a 1.7-fold increase in conjugated pigment synthesis when compared with control cells [45]. These increases agree well with the current data on UDP-N-AG-activated enzyme determinations. The analysis of expression of the different isoforms of UGT by Western and Northern blotting contributed to clarify the molecular basis of changes in UGT activities occurring in response to SL. However, the magnitude of these changes does not necessarily apply to the *in vivo* situation. Taken together, these results demonstrate that *in vivo* glucuronidation is modulated not only by UGT gene expression but also by the different steps involved in the whole process including uptake/secretion of substrates and/or products into/from the luminal catalytic site.

No clinical studies are available in the literature about alterations caused by SL administration in the metabolism of the UGT substrates studied here. The only report in humans refers that simultaneous administration of SL shortens the half-life of digitoxin, a cardiac glycoside, by 20% [46]. In rats, administration of the steroid at a similar dosage used in the current study, produced a 4- to 6-fold increase in liver microsomal UGT activity toward digitoxigenin monodigitoxoside [47], a major metabolite of digitoxin [48]. Thus, it is possible that SL-induced accelerated biotransformation of digitoxin in humans results, at least in part, from increased glucuronidation of one of its major derivatives. Digitoxigenin monodigitoxoside is mainly conjugated by isoforms of family 1 of UGT other than those involved in bilirubin and *p*-nitrophenol glucuronidation, both in human and rodents [49–53]. Further studies are necessary to demonstrate the induction of liver UGT1A1, 1A5 and 2B1 by SL in humans and the eventual clinical relevance of these findings as extensively described for phase I enzymes [19].

In conclusion, we demonstrated upregulation of UGT1A1, 1A5 and 2B1, but not 1A6, by SL. This agreed well with increased glucuronidation rate of bilirubin and ethynylestradiol (both in position 3-OH and 17β-OH). Northern blot studies revealed that regulation of UGTs by SL was at least partially at transcriptional level.

Acknowledgments

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