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Biochemical Pharmacology 63 (2002) 1683–1690

Biochemical Pharmacology

Tissue-specific regulation of canine intestinal and hepatic phenol and morphine UDP-glucuronosyltransferases by β-naphthoflavone in comparison with humans

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Received 29 November 2001; accepted 21 February 2002

Abstract

UDP-glucuronosyltransferases (UGTs) are regulated in a species- and tissue-dependent manner by endogenous and environmental factors. The present study was undertaken to further our knowledge about regulation of UGTs in dogs, a species widely used in preclinical safety evaluation. β-Naphthoflavone (BNF) was selected as a known aryl hydrocarbon receptor agonist and antioxidant-type inducer. The latter group of inducers is intensively investigated as dietary chemoprotectants against colon cancer. Dog UGTs were investigated in comparison with related human UGTs by examples, (i) expression of dog UGT1A6, the first sequenced dog phenol UGT, and (ii) morphine UGT activities, responsible for intestinal and hepatic first-pass metabolism of morphine. The following results were obtained: (i) dog UGT1A6 was found to be constitutively expressed in liver and marginally increased by BNF treatment. Expression was low in small intestine but ca. 6-fold higher in colon than for example in jejunum. Conjugation of 4-methylumbelliferone, one of the substrates of dog UGT1A6, was also enhanced 7-fold in colonic compared to jejunal microsomes. (ii) Compared to the corresponding human tissues, canine 3-O- and 6-O-morphine UGT activities were found to be >10-fold higher in dog liver and ca. 10-fold lower in small intestinal microsomes. Small intestinal morphine and 4-hydroxybiphenyl UGT activities appeared to be moderately (2- to 3-fold) induced by oral treatment with BNF. (iii) In contrast to dogs, morphine UGT activities were found to be similar in homogenates from human enterocytes and liver. The results suggest marked differences in tissue-specific regulation of canine vs. human hepatic and intestinal phenol or morphine UGTs.

Keywords: UDP-glucuronosyltransferase; Dog UGT1A6; UGT activity; Liver and intestine; β-Naphthoflavone; Morphine glucuronidation

1. Introduction

Glucuronidation plays a central role in phase-II biotransformation by converting hundreds of lipid-soluble endogenous compounds such as bilirubin and steroids, as well as xenobiotic drugs and dietary plant constituents into biologically inactive, hydrophilic and excretable conjugates [1]. This reaction is catalysed by a supergene family of UDP-glucuronosyltransferases (UGTs) which are typical membrane proteins of the endoplasmic reticulum and the nuclear

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Abbreviations: AhR, aryl hydrocarbon receptor; BNF, β-naphthoflavone; EDTA, ethylenediamine tetraacetic acid; EROD, ethoxyresorufin-O-dealkylase; M3G, morphine-3-O-glucuronide; M6G, morphine-6-O-glucuronide; PROD, pentoxyresorufin-O-dealkylase; UGT, UDP-glucuronosyltransferase.

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PII: S0006-2952(02)00921-8

envelope. Based on evolutionary divergence mammalian UGTs have been grouped into two distinct families [2]. Interestingly, all UGT1 members share the same last four exons with first exons distinguishing them. The gene organisation of the UGT1A locus appears to be conserved in mammalian species. Knowledge about regulation of UGTs is still insufficient. UGTs are regulated by tissue-specific endogenous factors [3,4] and by prototypical inducers of drug metabolising enzymes such as aryl hydrocarbon receptor (AhR) agonists and phenobarbital [5]. In addition, UGTs are known to be regulated by a chemically diverse group of 'antioxidant-type inducers' which trigger an adaptive response leading to transcriptional activation of phase-II genes (glutathione-S-transferases, NADPH quinone oxidoreductase-1, UGTs etc.; [6]) and to down regulation of CYP1A1 [7]. When administered in subtoxic concentrations the adaptive response enables the exposed cells to survive the toxicity of antioxidant-type inducers. It is conceivable that antioxidant-type inducers act preferentially in the intestinal epithelium, the first defence barrier to orally administered chemicals. β-Naphthoflavone (BNF) was selected as a prototypical inducer which combines the actions of AhR agonists and antioxidant-type inducers. Antioxidant-type inducers including dietary flavonoids, isoflavonoids and drugs such as Oltipraz are intensely studied in the efforts of chemoprotection against colon cancer [8–12].

Preclinical safety evaluation of drugs usually is carried out with laboratory species, for example rodents and nonprimate species such as the dog. Induction of drug metabolising enzymes such as UGTs can markedly change the pharmacokinetics of xenobiotics and thereby alter their pharmacological or toxicological effects. However, knowledge about the regulation of dog UGTs is scarce. To further our knowledge about dog UGTs we studied: (i) expression of the first characterised dog UGT1A6 [13]. Regulation of orthologous UGT1A6 isoforms has already been studied in rats [14,15], rabbits [16] and human cell models [17], and marked species differences have been observed. For example, there is low basal expression in rat liver while UGT1A6 is inducible >10-fold by AhR agonists. In contrast, high constitutive expression of this isoform has been found in rabbit liver, with no further induction by AhR agonists. (ii) Morphine UGT activities were studied since a large database exists about the pharmacokinetics and glucuronidation of this important drug [18]. A canine phenobarbital-inducible morphine UGT has previously been purified [19]. In addition to formation of the inactive morphine-3-glucuronide (M3G), morphine is also conjugated to morphine-6-glucuronide (M6G) which is a more potent analgesic than morphine itself [20,21]. Formation of both M3G and M6G is catalysed by UGT2B7, a major isoform of family 2, which is expressed in a variety of tissues including human brain [22,23]. In human Caco-2 cells UGT2B7 has previously been shown to be enhanced by antioxidant-type inducers [17]. Hence, phenol UGT1A6 and morphine UGT activities appear to be well suited for comparative studies between dogs and humans.

2. Materials and methods

2.1. Animal treatment and preparation of tissue microsomes

Adult male Beagle dogs (16–18 kg) were fed Specific CXD chow; three dogs were untreated, two dogs (BNF 1 and BNF 2) were treated with BNF (50 mg/kg per os for 5 days; BNF was dissolved in PEG 400), one dog (PB 1) was treated with phenobarbital (10 mg/kg for 6 days) as described [24]. Before removal of tissues the animals were anaesthetised with sodium pentobarbital intravenously and euthanised by exsanguination. Tissue samples from liver, kidney, duodenum, jejunum, ileum, colon, rectum and brain were removed quickly and shock-frozen in liquid nitrogen. The study was approved by the Stockholm Södra Animal Research Ethical Board. Microsomal fractions were obtained by standard techniques. Intestinal microsomes were obtained in the following way: the entire length of the intestine was removed and rinsed with ice-cold 0.15 M KCl. The intestine was then divided into the duodenum (10 cm), jejunum (130 cm), ileum (3 cm) and colon down to the rectum. Sections of the intestine were opened longitudinally and the mucosa was gently removed by scraping the inside of the intestine with a metal slide. Mucosal samples were pooled and frozen in liquid nitrogen. After thawing on ice, homogenisation of the mucosa was carried out in Tris-buffered 0.25 M sucrose containing 0.5 mM ethylenediamine tetraacetic acid (EDTA) and phenylmethylsulfonyl fluoride (40 μg/mL). After centrifugation at 10,000 *g* for 20 min, the supernatant was further centrifuged at 105,000 *g* for 1 hr. The pellet was washed in 0.15 M KCl and resuspended in 0.05 M phosphate buffer, pH 7.4.

2.2. Human tissue bank and enterocyte preparation

Intestinal tissue without pathology was obtained from 12 surgical preparations and liver biopsies from the same patients. The preparations were obtained with the informed consent of the patients and approval of the ethics committee. Six male and six female patients (37–78 years) were included; patients with inflammatory diseases were excluded.

Deep frozen intestinal tissue was warmed up in two steps, slowly till -20° and rapidly till 4°. Enterocytes were obtained using a vibration method in the presence of EDTA, as described [25]. Morphological and immunohistochemical controls revealed isolated villus epithelium without crypts and complete villi. Villus epithelium was identified with antibodies against villin. The isolated villus epithelium was homogenised in the presence of protease inhibitors including leupeptin, pepstatin and EDTA.

2.3. Enzyme assays

2.3.1. CYP activities

Ethoxyresorufin-O-dealkylase (EROD) and pentoxyresorufin-O-dealkylase (PROD) were determined as described [26–28].

2.3.2. UGT activities

Described methods were used for the assay of UGT activities towards 4-methylumbelliferone and 4-hydroxybiphenyl [29]. For reasons of standardisation and comparison, the assays were performed at 37° in the presence of 0.1 M Tris–HCl, pH 7.4 and 5 mM $MgCl_2$ in 0.5 mL incubation medium. UGT activities in microsomal fractions were fully activated by addition of the detergent Brij 58 or the pore forming polypeptide alamethicin (Fig. 1). Alamethicin was preferred since detergents (i) must be titrated to determine optimal conditions for full activation, and (ii) interfere with LC-MS determination of glucuronides [30]. Alamethicin, 1.5 mg/mg protein of dog liver microsomes corresponded to 30 μg/mL incubation medium. It was found that the optimal alamethicin based on milligram protein varies considerably corresponding to the UGT activity, but variation is small when the optimal alamethicin concentration per millilitre incubation medium is given. Therefore we activated UGT activity by addition of 30 μg alamethicin/mL incubation medium for all UGT activities and tissues. In the assay of morphine UGT activities M3G and M6G were separated by LC-MS with deuterated analogues as internal standards [31].

2.4. RT-PCR of dog UGT1A6 mRNA and of all dog UGT1A isoforms

Total RNA from dog tissue was isolated using trizol purchased from Invitrogen. Primers for RT-PCR were selected by alignment of UGT1A6 sequences of human (Acc. no. JO 4039.1), rat (Acc. no. U 20551.1) and dog (Acc. no. AJ 290948) [13] orthologous isoforms using BLASTN software. Exon-intron junctions were localised comparing the complete sequences of UGT1A6 (Acc. no. AF 297093) to UGT1A6 cDNA (Acc. no. JO 4039.1) and aligned to dog cDNA UGT1A6 [13] using ALIGN software. Primers for dog UGT1A6 were selected in analogy to human UGT1A gene organisation: dog UGT1A6 forward primer: nt 706–735 5′-GATGTGCACTTACCCACCTTGTATCGGAAC-3′ and reverse primer (presumed exon 2) from nt 908–941 5′-GCTTTCCTTCTCAGGAATATCTGATACCATGGAG-3’ spanning the presumed intron 1. All dog UGT1A isoforms (presumed exons 3–5): forward primer nt 991–1017 5′-CGTTATACTGGCACTCCACCACCGAAT-3′ and reverse primer nt 1503–1533 5′-CTTCCGGCAGCCAAAGGCACAACATTTGTAA-3′. RT-PCR was performed by 26 cycles for liver samples and by 34 cycles for the other tissue samples. After heating the master mix containing the RNA-derived cDNA samples [17] and Taq DNA polymerase to 94° for 30 s, annealing was performed at 55° for 30 s and extension at 72° for 1 min. Thereafter a 10 min elongation step at 72° was included. PCR products were separated by polyacrylamide gel electrophoresis using 6% native gels and evaluated by direct phosphorimage analysis using the Fuji Bio-Image BAS1500 analyser. Standard curves with different amounts of cDNA were used to verify that the cycle number was optimal for quantification. Sequence analysis of PCR products of dog UGT1A6 and of the common exons 3–5 of all dog UGT1 isoforms indicated, that the published dog UGT sequences were amplified [13].

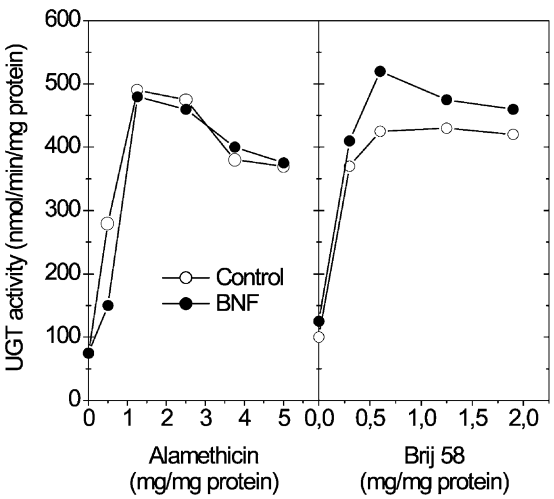

Fig. 1. Activation of canine hepatic microsomal 4-methylumbelliferone UGT activity by alamethicin and Brij 58. A representative experiment is shown.

2.5. Northern blot analysis

It was performed as described [32]. The UGT1A6 RT-PCR products from dog liver mRNA (236 bp) were labelled using random primed labelling kit (Roche) and used as probes. Membranes were washed and analysed by phosphorimaging. Ethidium bromide staining was used to control equal loading of the lanes.

2.6. Statistics

For the assessment of treatment effects in Figs. 2 and 4 analysis of variance of log-transformed data was used.

3. Results

3.1. Tissue-specific expression and regulation of dog UGT1A6

Sequences of dog and human UGT1A6 isoforms were 78.9% identical for the presumed dog exon 1 encoding the N-terminal part of the isoform; it was 86.7% for exons 2–5 encoding the C-terminal part. Therefore it was assumed

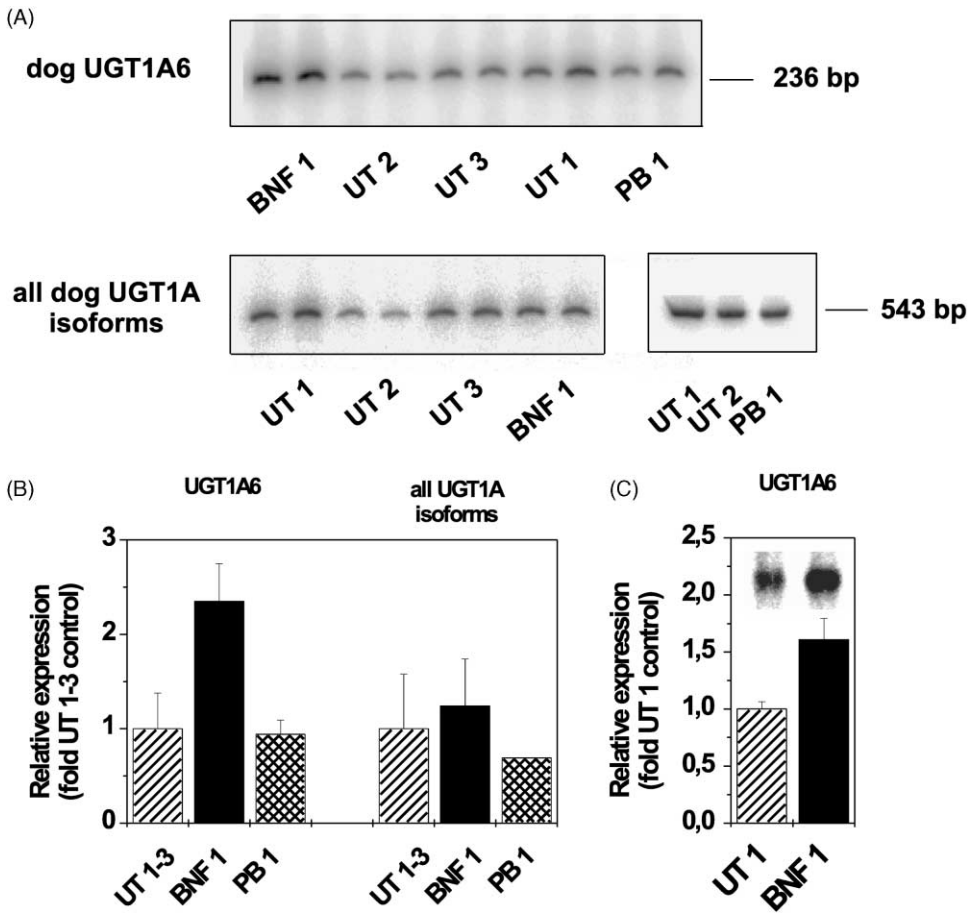

Fig. 2. (A and B) Expression of dog hepatic UGT1A6 and of all UGT1 isoforms, detected from the common exons of the UGT1 gene. The specificity of RT-PCR products was confirmed by direct sequencing. RNA was isolated from shock-frozen livers of three untreated dogs (UT 1–3), of one BNF-treated dog (BNF 1) and of one phenobarbital-treated dog (PB 1). (C) Northern blots were carried out using the specific UGT1A6 RT-PCR product. For each dog UGT expression was measured by four independent determinations. Means ± SD are listed.

that the organisation of the UGT1A locus in mammalian species was conserved and that dog UGT1A6 expression was measured selectively by amplification of exon 1 whereas amplification of exons 3–5 yielded information about the expression of all UGT1A isoforms.

Dog UGT1A6 was constitutively expressed in liver with marginally increased expression (2-fold; $P = 0.074$) in the BNF-treated dog but not in the phenobarbital-treated dog (Fig. 2A and B). Increased expression was also seen when comparing mRNA from BNF 1 and the untreated dog UT 1 by northern blot analysis (Fig. 2C). No change in UGT expression was observed when all UGT1A isoforms were measured by amplification of exons 3–5. Expression of dog UGT1A6 was also detectable in extrahepatic tissues. It was much lower in the small intestine than in liver but ca. 6-fold higher in dog colon than in the jejunum (Fig. 3). Interestingly, when the expression of all UGT1 isoforms was measured, UGT expression appeared to be similar in liver and intestine.

UGT1A6 represents a phenol UGT conjugating planar phenols such as 4-methylumbelliferone or 1-naphthol. In line with dog UGT1A6 expression, 4-methylumbelliferone UGT activity was high in dog liver microsomes, low in small intestinal microsomes, but ca. 9-fold higher in colonic than in jejunal microsomes (Figs. 4 and 5). Low 4-methylumbelliferone UGT activity was also detectable in the microsomal fraction of kidney and brain (0.50 ± 0.06 and 0.14 ± 0.05 nmol/min/mg protein, respectively).

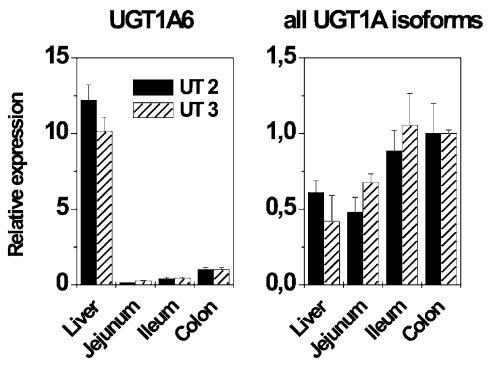

Fig. 3. Expression of canine hepatic and intestinal UGT1A6 and of all UGT1A isoforms. RNA was isolated from shock-frozen tissues of two untreated dogs (UT 2 and UT 3). Means ± SD of three determinations are listed.

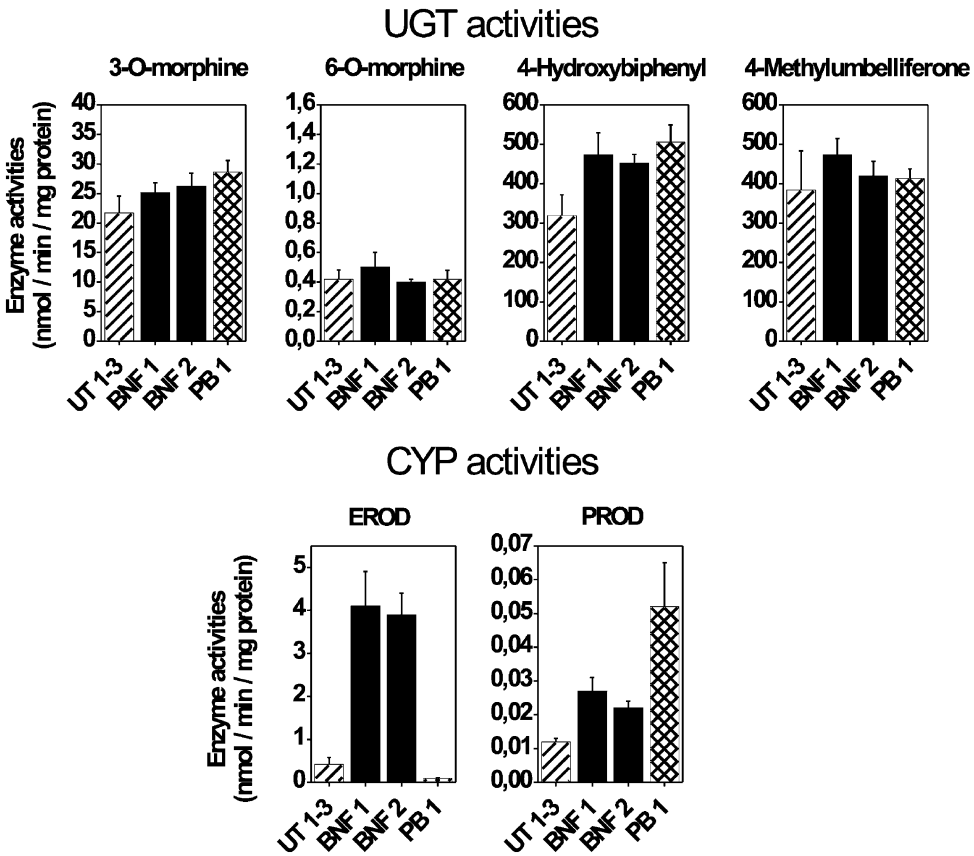

Fig. 4. Canine hepatic UGT and CYP activities expression and their regulation by BNF and phenobarbital. Enzyme activities were determined in hepatic microsomes, UGT activities in alamethicin-activated microsomes. Means ± SD of four determinations (each) from three untreated dogs (UT 1–3), two BNF-treated dogs and one phenobarbital-treated dog are compared.

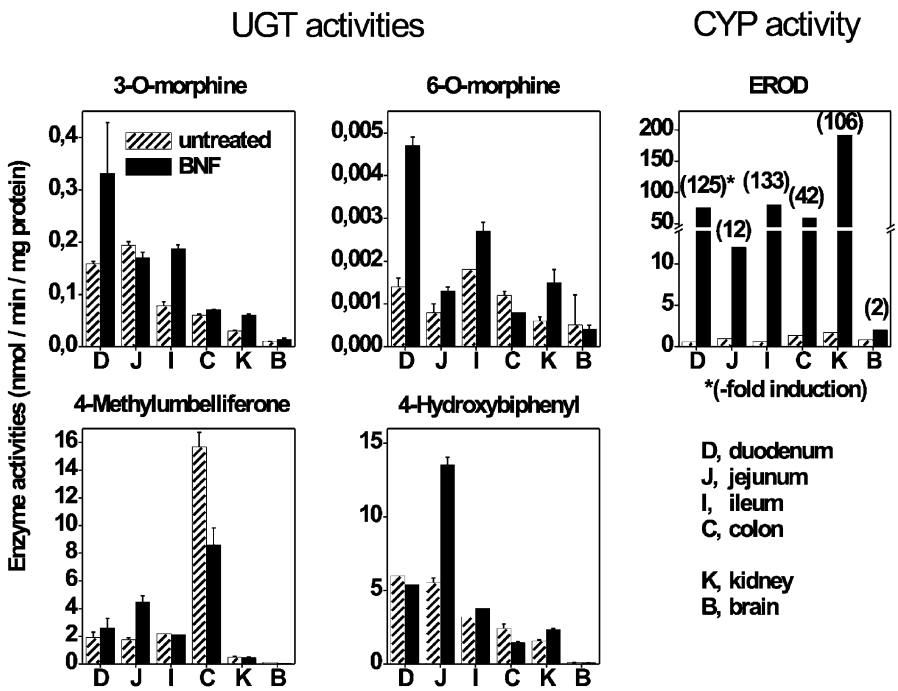

Fig. 5. Canine intestinal as well as renal and brain UGT and CYP activities and their regulation by BNF. Determinations were carried out with tissue microsomes from one untreated dog (UT 1) and one BNF-treated dog (BNF 1). Means ± SD of three determinations are shown.

3.2. Regulation by BNF of canine hepatic and intestinal morphine UGT activities

Both M3G and M6G formation were clearly detectable in dog liver microsomes (Fig. 4). UGT activities toward morphine were not induced by BNF but marginally enhanced in phenobarbital-treated dog liver microsomes (ca. 1.5-fold), in agreement with [19]. Hepatic UGT activity toward 4-methylumbelliferone was not enhanced by the two inducers, but UGT activity toward 4-hydroxybiphenyl was moderately enhanced by both BNF and phenobarbital ($P = 0.056$). Marked and differential induction of the CYP activities EROD and PROD in liver microsomes indicated that oral treatment with BNF and phenobarbital according to [24] was effective (Fig. 2). EROD and PROD activities were selectively enhanced in BNF- and phenobarbital-treated dogs, respectively.

It is conceivable that regulation of UGTs by dietary inducers occurs primarily in the intestine. EROD activity was hardly detectable in the intestine of untreated dogs but was strongly enhanced (>10- to >100-fold) in various intestinal sections of the BNF-treated dog investigated (Fig. 5), and these findings were confirmed in another BNF-treated dog (not shown). Low M3G and M6G formation was also detectable in intestinal microsomes. Interestingly, UGT activities towards morphine and 4-hydroxybiphenyl appeared to be moderately enhanced (ca. 2-fold) in small intestinal microsomes of the BNF-treated dog, in contrast to the colon. The reason why CYP1A1 activity and morphine UGT activities were preferentially increased in duodenum and ileum whereas 4-hydroxybiphenyl UGT activity was enhanced in jejunal microsomes is unknown. Low UGT activities could also be detected in dog kidney and brain: UGT activities toward 4-hydroxybiphenyl were 1.59 ± 0.04 and 0.10 ± 0.01, toward 3-O-morphine 0.031 ± 0.001 and 0.012 ± 0.001 nmol/min/mg protein, and toward 6-O-morphine 0.6 ± 0.1 and 0.5 ± 0.1 pmol/min/mg protein, respectively.

3.3. Comparison of human and canine hepatic and intestinal morphine UGT activities

Quantitative comparison of hepatic and intestinal UGT activity is difficult, not the least because of different isolation procedures of intestinal microsomes. While dog intestinal microsomes were obtained from mucosal scrapings, human intestinal homogenates were obtained from enterocyte preparations, similar to previous studies with rat intestinal microsomes [25]. Interestingly, human hepatic and intestinal morphine UGT activities appear to be similar, in contrast to ca.10-fold higher hepatic and 10-fold lower intestinal activities in dogs and rats (Table 1). From UGT activities in human homogenates, microsomal activities were estimated by multiplication with suitable conversion factors. In a parallel study conversion factors of 2.5 and 1.8 were obtained by determining CYP3A4 activities in the same homogenates and in microsomes.[1] Multiplying homogenate UGT activities with these factors leads to microsomal morphine 3-O-UGT activity of 2.3 and 1.5 nmol/min/mg protein for hepatic and intestinal microsomes, respectively. Hence, the above conclusions remain valid when microsomal activities are compared. Ratios of M3G to M6G were different in hepatic and intestinal microsomes suggesting involvement of more than one UGT in M3G and M6G formation.

Table 1
Species comparison of hepatic and intestinal morphine UGT activities in hepatic and intestinal microsomes

Tissue	Morphine UGT activities (nmol/min/mg protein)		
	Human	Rat[a]	Dog
Liver			
M3G	0.93 ± 0.21[b]	5.8 ± 0.6	22 ± 3[c]
M6G	0.15 ± 0.03	n.d.	0.4 ± 0.1
Intestine			
M3G	0.82 ± 0.10	0.07 ± 0.01	0.13
M6G	0.06 ± 0.01	n.d.	0.001

n.d., not detectable.

[a] Taken from [25]. Data represent means ± SEM (N = 4).

[b] Data represent means ± SD obtained with homogenates of liver biopsies and enterocytes from 12 individuals.

[c] Means ± SD of four and two determinations, respectively, with liver and jejunal microsomes from two untreated dogs are listed.

4. Discussion

Tissue-specific expression of canine UGTs and their regulation by BNF has been studied, (i) expression of the first sequenced dog phenol UGT1A6 and of all dog UGT1 isoforms (detected from the common exons), and (ii) morphine UGT activities catalysed by unknown dog UGT isoforms.

4.1. Tissue-specific regulation of dog UGT1A6

Sequence comparison of dog UGT1A6 [13] with rat and human UGT1A6 orthologues [2] suggests an evolutionary conserved structure of the UGT1 locus in which variable first exons were spliced to identical exons 2–5. Based on this assumption RT-PCR methods were developed to detect dog UGT1A6 expression by amplification of its exon 1 and the expression of all UGT1A isoforms by amplification of the identical exons 3–5. UGT1A6 mRNA was found to be constitutively expressed in dog liver and marginally induced by BNF treatment. No effect of BNF was seen on the expression of all UGT1A isoforms. High constitutive expression of dog liver UGT1A6 was supported by high 4-methylumbelliferone UGT activities, identified

[1] Oliver von Richter. Unpublished work.

previously as substrates of dog UGT1A6 [33]. An inverse relationship between high constitutive expression and inducibility of UGTs has been suggested before [15] and may explain poor induction in dog liver by BNF.

Low UGT1A6 expression was also detected in dog intestine. Interestingly, expression was 6-fold higher in colon than for example in jejunum, a finding in contrast to humans in which colonic UGT1A6 expression was low [34]. Amplification of the common UGT1A exons showed at least comparable expression in the intestine and liver, suggesting high expression of unidentified dog UGT1A isoforms in the intestine, similar to findings in humans [34]. High phenol UGT levels in dog colon were suggested by 7-fold higher 4-methylumbelliferone UGT activity in colon than in jejunum. The reason for the high capacity of the dog to detoxify planar phenols in the colon is unknown.

4.2. Regulation by BNF of canine hepatic and intestinal morphine UGT activities

Morphine represents an important drug substrate of UGTs since—in addition to formation of inactive M3G—M6G is formed which is more potent as an analgesic drug in brain than morphine itself [20,21]. It has been shown that both M3G and M6G are formed by human UGT2B7 [22]. This isoform has previously been shown to be inducible by antioxidant-type inducers [17]. Using a sensitive LC-MS method, M3G and M6G formation could be detected in dog liver at a ratio of ca. 50:1 (in agreement with [35]) and in the intestine. Previously, the intrinsic clearance of morphine was found to be >10-fold higher in dog than in human liver microsomes [33]. In line with its high constitutive expression, BNF treatment did not enhance morphine UGT activities in liver. Evidence for moderate induction (ca. 2- to 3-fold) of morphine, 4-hydroxybiphenyl and 4-methylumbelliferone UGT activities by oral BNF administration was observed in small intestines of dog but not in colonic microsomes, suggesting that several UGTs are regulated by antioxidant-type inducers in dog intestinal epithelium similar to findings in human Caco-2 cells [17]. Intestinal CYP1A1 activity was strongly induced by BNF. This cytochrome is believed to metabolise BNF to electrophilic phenolic radicals which may generate electrophile stress and trigger the antioxidant-type induction of phase-II enzymes [6]. Although moderate induction of intestinal UGT activities appears conceivable the findings have to be considered preliminary due to the small sample size.

4.3. Comparison of human and dog hepatic and intestinal morphine UGT activities

Hepatic and intestinal tissues from a tissue bank of a carefully selected group of 12 patients were used to determine human morphine UGT activities, in comparison with the corresponding dog activities. In contrast to our findings of low intestinal UGT activity compared to liver in dogs and rats, human morphine UGT activities were found to be similar in these two tissues [25]. These species differences may have pharmacokinetic implications. UGTs in the small intestine are part of the first defence barrier against dietary toxins [34]. Moreover, it is increasingly recognised that the fate of glucuronides is determined by glucuronide transporters or multidrug resistance proteins (MRPs) [36,37]. In polarised cells such as hepatocytes and enterocytes MRP2 and MRP3 are localised at the apical and basolateral surfaces, respectively, and therefore determine the disposition of glucuronides. First-pass metabolism studies usually measure the contribution of both liver and intestine. High constitutive UGT expression and UGT activities in dog liver support high first-pass metabolism by glucuronidation in dogs. More work is needed to differentiate the separate contributions of intestinal epithelium and liver to first-pass metabolism in dogs and humans and to the local protection against dietary toxins.

Acknowledgments

We thank Dr. Ute Hofmann for LC-MS analysis of morphine glucuronides, Ulla-Britt Gunnarsson and Pia Holmquist as well as I. Voith and B. Rudo for expert technical assistance, Dr. K. Dietz (Department of Medical Biometry, University of Tübingen, Germany) for carrying out the analysis of variance, as well as the Deutsche Forschungsgemeinschaft and AstraZeneca for financial support.

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