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Research Paper

Repeated administration of oxycodone modifies the gene expression of several drug metabolising enzymes in the hepatic tissue of male Sprague-Dawley rats, including glutathione *S*-transferase A-5 (rGSTA5) and CYP3A2

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Abstract

Objectives Clinical use and illicit abuse of the potent opioid agonist oxycodone has dramatically increased over the past decade. Yet oxycodone remains one of the least studied opioids, particularly its interactions on the genomic level. The aim of this study was to examine potential alterations in gene expression of drug metabolising enzymes in the liver tissue of male Sprague-Dawley rats chronically treated with oxycodone.

Methods Rats were administered saline or oxycodone 15 mg/kg i.p. twice a day for 8 days. Changes in RNA levels were detected using microarray analysis validated by quantitative real-time PCR; consequent changes in protein expression and functionality were further assessed by Western blotting and activity assays.

Key findings The expression of several drug metabolising enzymes was modulated by oxycodone treatment: cytochrome P450 (CYP) 2B2, CYP2C13, CYP17A1, epoxide hydrolase 2, carboxylesterase 2, flavin-containing monooxygenase 1, glutathione *S*-transferase alpha 5 (rGSTA5) and CYP3A2. In particular, the mRNA level of rGSTA5 (formerly $GSTYc_2$) was up-regulated by approximately 6.5 fold and CYP3A2 was down-regulated by approximately 7.0 fold. Immunoblotting assays demonstrated a corresponding significant elevation of rGSTA5 protein and repression of CYP3A2 protein. The apparent cytosolic GST activity towards 1-chloro-2,4-dinitrobenzene conjugation and reduction of cumene hydroperoxide were significantly higher in liver from oxycodone-treated rats than that of saline-treated rats. In addition, the microsomal activity of CYP3A2, measured via 6β-hydroxylation of testosterone, was significantly decreased in oxycodone-treated rats.

Conclusions Repeated oxycodone administration is associated with a significant up-regulation of rGSTA5 and concomitant down-regulation of CYP3A2 mRNA, protein expression and functionality. These results support further in-vivo studies into the clinical impact of our findings.

Keywords rGSTA5; CYP3A2; microarray; oxycodone; drug metabolising enzymes

Introduction

Oxycodone (14-hydroxy-7,8-dihydrocodeinone) is a semi-synthetic opioid derivative of thebaine and is structurally similar to morphine and other phenanthrene opium alkaloids. It is extensively metabolised via *N*- and *O*-demethylation, 6-ketoreduction and glucuronidation.[1] Several of these metabolic pathways proceed via cytochrome P450 (CYP)-mediated oxidation, including *O*-demethylation to oxymorphone, a potent but quantitatively minor metabolite,[2] and *N*-demethylation to the inactive metabolite noroxycodone.[3] CYP2D6 has been identified as the high-affinity enzyme responsible for *O*-demethylation, and CYP3A4 was shown to be the high-affinity CYP isoform involved in *N*-demethylation.[4]

Documentation of clinical oxycodone use dates back to 1917.[5] Historically, it was most commonly prescribed in the USA to treat mild-to-moderate pain in low-dose combinations with paracetamol or aspirin.[5] In 1995, the US Food and Drug Administration approved a sustained-release formulation containing solely oxycodone hydrochloride (OxyContin). Since then, annual prescriptions in the USA have increased markedly.[6,7] The amount of

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oxycodone prescribed or dispensed in the USA increased by over 1000% from 1999 to 2002.[8] Specifically, use of oxycodone for moderate-to-severe cancer-related pain has increased greatly in recent years.[5]

In addition to analgesia, oxycodone administration produces pleasurable, euphoric and reinforcing effects that are primarily mediated by mu-opioid receptors,[9] which commonly leads to misuse and abuse.[10] Although reports of abuse were anecdotal at first, reports on epidemic-type increases in oxycodone abuse have recently been published.[11–13] Dasgupta *et al.*[8] found a substantial correlation between the number of emergency department mentions recorded by the Drug Abuse Warning Network (DAWN) and oxycodone prescription use. Another DAWN-based categorisation system found nearly 1000 oxycodone-related deaths from 1999 to 2002 in 23 states, in which over 90% of the cases were due to multiple drug abuse or polypharmacy related deaths. The Institute for Safe Medication Practices has included oxycodone in the drug class with a heightened risk of significant harm when used in error (www.ismp.org).

Despite the well documented and widespread clinical use and illicit abuse of oxycodone, little is known about its potential effects on the genomic regulation of common drug transporters and drug metabolising enzymes (DMEs). In previous studies we studied the effects of oxycodone treatment on the major drug efflux transporter P-glycoprotein (P-gp). We established that chronic administration of oxycodone to male Sprague-Dawley rats resulted in the induction of P-gp protein in the liver, kidney, intestine and brain.[14] Furthermore, brain uptake studies following a single intraperitoneal dose of radiolabelled paclitaxel showed a significant reduction in brain concentrations of paclitaxel, suggestive of a potential clinical drug–drug interaction between this drug and oxycodone via P-gp.[14] In another recent study, we used a microarray approach to demonstrate that chronic administration of oxycodone significantly modulated a group of genes in rat brain tissues, including the efflux transporter Abcg2 (breast cancer resistance protein; BCRP).[15] Subsequent brain uptake studies demonstrated that oxycodone treatment resulted in a significant decrease in brain/plasma ratios of mitoxantrone, also suggestive of a clinical drug–drug interaction.[15]

In the current study we build upon our current global microarray analysis of liver tissues, focusing on alterations in gene expression of DMEs in the liver of male Sprague-Dawley rats treated with oxycodone or saline (control). To identify up- or down-regulated genes, we performed microarray and quantitative real-time (QRT) PCR analyses. We used Western blotting and several functional assays to further characterise changes in protein expression and functional implications of two particular DMEs – rat glutathione *S*-transferase A-5 (rGSTA5) and CYP3A2, both of which are involved in the metabolism of a diverse range of endogenous and exogenous substrates.[16–18]

Materials and Methods

Materials

Oxycodone (14-hydroxy-7,8-dihydrocodeinone) was graciously donated by Dr Andrew C. Coop (University of Maryland). Cumene hydroperoxide 88% (CuOOH), 1-chloro-2,4 dinitrobenzene (CDNB), glutathione (GSH), glutathione reductase from baker's yeast (*Saccharomyces cerevisiae*), β-NADPH, EDTA and sodium azide were all purchased from Sigma-Aldrich Co. (St Louis, MO, USA). All other chemicals, solvents and reagents were of analytical grade or highest purity available.

In-vivo study

Male Sprague-Dawley rats (270–300 g) purchased from Harlan Laboratories (Indianapolis, IN, USA) were separated into two groups of six. The rats were housed individually and allowed to acclimate to their new environment at least 2 days prior to the experiments. They were provided with standard rat chow and tap water *ad libitum* and maintained under a standard 12-h light–dark cycle.

The protocol for the animal studies was approved by the University of Maryland, School of Pharmacy, Institutional Animal Care and Use Committee.

Rats were given either oxycodone 15 mg/kg i.p. twice daily or saline 1 ml/kg i.p. twice daily for 8 days. The mean daily oxycodone dose (8.25 ± 1 mg) administered in this study is pharmacologically relevant and was based on previous literature. For example, patients with non-cancer related chronic pain receive oxycodone doses in excess of 275 mg/day,[19] and the average daily oxycodone dose in cancer patients was 150 ± 20 mg daily.[20]

On the morning of the 9th day rats were asphyxiated using carbon dioxide. Liver tissues were harvested immediately and placed on ice.

RNA isolation

Total RNA was isolated using the Trizol reagent, according to the manufacturer's standard protocol (Invitrogen, Carlsbad, CA, USA). Harvested liver tissue was homogenised in Trizol reagent, followed by organic extraction and ethanol precipitation. RNA concentration was quantified by UV spectroscopy at 260 nm and purity gauged by determining the 260/280 nm ratio. Thirty micrograms of isolated RNA was treated with DNase for 10 min using a DNase kit (Qiagen Inc., Carlsbad, CA, USA), according to the manufacturer's standard protocol, and was further purified using the RNeasy MinElute Kit (Qiagen Inc.). The concentration of purified RNA was measured using spectroscopy. RNA quality was assessed by electrophoresis on a 1% agarose gel, and using the 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA). Only total RNA with sharp and well-defined 28S and 18S ribosomal RNA peaks, indicative of acceptable RNA integrity, was used for further analysis.

Expression profiling

Gene profiling was performed using the GeneChip rat genome 230 2.0 array (Affymetrix, Santa Clara, CA, USA) containing 31 000 gene probe sets. Each treatment group (oxycodone and saline) consisted of three sets of RNA, each set derived from pooled RNA from two rats. RNA samples in each set were used to make templates for in-vitro transcription; the labelled transcripts were prepared using the one-cycle target labelling and control reagent kit using the

Affymetrix protocols. Standard conditions were used for hybridisation; washing was with the Affymetrix Fluidics Station 450 and Hybridization Oven 640. Array scanning was performed with the Affymetrix GeneChip Scanner 3000. RNA labelling, hybridisation, washing and scanning were conducted at the University of Maryland, School of Medicine, Biopolymer/Genomics Core Facility.

Microarray data analysis

RNA quantification was performed using the background-adjusted robust multiple average method and implemented in the Bioconductor R packages *Affy* and *gcrma*.[21,22] The detection call *P* value calculated by Affymetrix Microarray suite was used as a marker of gene expression (present, marginal or absent). Fold change in the transcript levels and statistical analysis were calculated using Spotfire DecisionSite 8.2.1 algorithm (Spotfire, Inc., Somerville, MA, USA). Results were tabulated into a spreadsheet and genes exhibiting significant up- or down-regulation were sorted separately from the remaining genes. The sorted list was scanned across numerous iterations for the presence of DMEs.

Quantitative real-time PCR

RNA levels of 10 DME genes (Table 1) were additionally examined by QRT-PCR to validate the microarray findings. The QRT-PCR assay was conducted using the iCycler iQ instrument according to the manufacturer's suggested protocol (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Briefly, cDNA was synthesised from 1 μg total RNA using the iScript cDNA Synthesis Kit (Bio-Rad). An aliquot (10%) of the reverse transcription reaction was used as the template for QRT-PCR reactions containing iQ SYBR Green Supermix (Bio-Rad) and a final primer concentration of 200 nmol/l. Primers (Table 2) were designed using the Beacon Designer 3.0 software (Premier Biosoft, Palo Alto, CA, USA). The basic local assignment search tool (BLAST) search engine (www.ncbi.nlm.nih.gov/BLAST) was used to test primer specificity against the whole rat genome database. QRT-PCR reactions included an initial denaturation step at 95ºC for 3 min, followed by 40 PCR cycles with a 30 s melt at 95ºC, then annealing and extension at 60ºC for 45 s. All reactions were performed in triplicate. The cycle threshold (Ct) was calculated automatically using the second derivative of the reaction. The Ct of each DME gene was normalised against the Ct for glyceraldehyde 3-phosphate dehydrogenase (GAPDH), which showed no significant regulation in our microarray analysis. Fold change was determined using the delta delta Ct method. Statistical significance ($P < 0.05$) between treatment groups was evaluated using the Student's *t*-test.

Cytosol and microsome preparation

Liver cytosol[23] and microsome[24] fractions were isolated separately from pooled liver ($n = 4$) samples. Protein concentration was measured using the Bio-Rad DC protein assay (Hercules, CA, USA), using bovine serum albumin as a standard. Samples were stored at −80ºC until further analysis.

SDS-PAGE and Western blotting

SDS-PAGE and immunoblotting of rGSTA5 were performed as described previously[25] using the Laemmli discontinuous buffer system[26] in a 10% polyacrylamide gel with a 5% stacking gel. The positive control was hepatic S9 fraction from pooled male mice purchased from BD Biosciences (Woburn, MA, USA) since it contains an appreciable amount of mouse GSTA3 (formerly known as GST Ya_3) which has strong cross-reactivity with anti-rGSTA5 and similar electrophoretic properties to rGSTA5.[27] Bio-Rad Immun-Blot PVDF membranes (0.2 μmol/l) containing transferred protein were incubated overnight at 4ºC with antisera raised in rabbits against rGSTA5 (1 : 5000 dilution) that was graciously donated by Dr John D. Hayes (University of Dundee, Biomedical Research Centre, Dundee, Scotland). Immunoreactive protein was detected using the SuperSignal West pico chemiluminescent substrate kit (Pierce, Rockford, IL, USA) on a Bio-Rad ChemiDoc XRS imaging system. For the control, the same membrane was probed in an analogous

Table 1 Differential expression of drug metabolising enzymes on microarray and quantitative real-time PCR analyses in the liver tissue of male Sprague-Dawley rats administered repeated doses of oxycodone 15mg/kg i.p. or saline control

Gene Title	Gene symbol	Microarray fold change	QRT-PCR fold change
Up-regulation			
Glutathione *S*-transferase alpha-5	GSTA5	7.0 (0.001)	6.0 (0.01)
P450 oxidoreductase	POR	8.0 (0.003)	9.0 (0.01)
CYP3A1	CYP3A1	2.0 (0.03)	3.0 (0.001)
CYP2B2	CYP2B2	6.0 (0.008)	7.0 (0.01)
CYP17A1	CYP17A1	17.0 (0.004)	9.0 (0.007)
Epoxide hydrolase-2	EPHX2	3.0 (0.04)	3.0 (0.03)
Carboxylesterase-2	CES2	3.0 (0.0001)	3.0 (0.02)
Flavin monoxygenase-1	FMO1	4.0 (0.01)	5.5 (0.01)
Down-regulation			
CYP2C13	CYP2C13	2.0 (0.002)	5.0 (0.01)
CYP3A2	CYP3A2	7.0 (0.04)	6.0 (0.01)

Values are expressed as fold change (*P* value). Fold change (oxycodone/saline-treated rats) in transcript levels on microarray were calculated using Spotfire DecisionSite 8.2.1 algorithm and in transcript levels on QRT-PCR using the delta delta cycle threshold method. *P* values on microarray were generated from ANOVA calculated by Spotfire software, and on QRT-PCR were calculated using Student's *t*-test.

Table 2 Sense and antisense primers used in qualitative real-time PCR analysis generated from Beacon Designer software

Gene symbol	Sense (5′→3′ direction)	Antisense (5′→3′ direction)	Accession no.
GSTA5	GCCGATCTGGAGTTGATGGTT	TCAGCTTGTTGCCAACGAGAT	NM_001009920
POR	CTGCTGTCACCGCCAACC	TCTGAGATGTCCAACTCCAGGT	NM_031576
CYP3A1	AGAGGAGTAATTTGCTGACAGACC	GTGTGCGGGTCCCAAATCC	NM_173144
CYP2B2	CGGCGATTCTCTCTGGCTAC	GCAGTTCCTCCACCAAACATTG	XM_341808
CYP17A1	GCCATCTGCCTACACCTGG	TGACTTGACCCAAAAGAAATAGGC	NM_012753
CYP2C13	ACCCTGTGATCCCCAATTCATC	CGGACAATAATCAAGAAGTATAGGAAATAT	J02861
CYP3A2	GAATGCTTTTCTGTCTTCACAAACC	TTTACCAAAATGTCTCCATACTGTTCA	NM_153312
EPHX2	CTGCCCAGAGACTTCCTACTTG	ATGGCTTGGCTGAATETTTCACTTAT	NM_022936
CES2	GCCATCATGGAGAGTGGAGTG	TGAAGACCTTGTTAATGACCAGAATC	NM_133586
FMO1	GCCGAGTCTTTGATTCAGGGTA	TCATCATTCAGCACAGGCTCTC	NM_012792
GAPDH	CCCATCACCATCTTCCAGGAG	GTTGTCATGGATGACCTTGGC	NM_017008

Gene descriptions for the corresponding gene symbol are included in Table 1.

fashion for immunoreactive β-actin. Immunoblotting procedures for detecting CYP3A2 in microsomal fractions were similar to above using an anti-CYP3A2 antibody (Abcam; Cambridge, MA, USA); the positive control was pooled male rat liver microsomes (BD Biosciences).

Enzymatic activity assays

Two functional spectrophotometric studies were used to measure cytosolic GST activity. Firstly, the apparent cytosolic GSH conjugation of CDNB was measured using a spectrophotometer following the method of Habig *et al*.[28] Secondly, the apparent selenium-independent GSH peroxidase activity via NADPH oxidation was assessed using the method described by Howie *et al*.[23]

The intra-day and inter-day variability for both GST activity assays was $\leq 15\%$ (%CV). CYP3A2 activity, graciously measured by Dr James P. Hardwick (Northeastern Ohio Universities College of Medicine, Rootstown, Ohio, USA) was measured via 6β-hydroxylation of testosterone using silica thin-layer chromatography (TLC) and autoradiography.[29]

Statistical analysis

Hepatic expression of rGSTA5 and CYP3A2 protein, as well as CYP3A2 testosterone hydroxylase activity, were compared between oxycodone- and saline-treated rats using an unpaired *t*-test as described previously[30] using SigmaStat version 3.10 (Systat Software; Point Richmond, CA, USA). Two-way analysis of variance followed by Bonferroni's post-hoc *t*-test was used to evaluate the differences between treatment groups and substrate concentrations in both GST functional assays.[30] The threshold of significance was set at $P < 0.05$.

Results

Differential expression of drug metabolising enzyme genes on microarray

Total RNA was freshly isolated and purified from the liver tissue of oxycodone- and saline-treated rats on the morning of the ninth study day. RNA levels were evaluated using an Affymetrix microarray rat genome array that contained approximately 31 000 probe sets that correspond to 28 000 well-substantiated rat genes. From the several hundred genes which showed altered expression, ten DME genes (Table 1), including those encoding isoenzymes from the GSTs, CYPs, epoxide hydrolases, carboxylesterases and flavin-containing monooxygenases, were identified. Eight out of ten (80%) of the differentially regulated genes were significantly up-regulated (Table 1), whereas two enzymes, CYP2C13 and CYP3A2, were significantly down-regulated.

Microarray validation using QRT-PCR

All ten of the DME genes showing differential expression on microarray were also subjected to QRT-PCR. Randomly selected genes ($n = 8$) showing no significant regulation on microarray were also evaluated by QRT-PCR to confirm the reliability of the statistical approach used for microarray data analysis (data not shown). Fold change and subsequent *P* values derived from the QRT-PCR analysis are shown in Table 1 alongside the microarray data for comparison. These results demonstrated a high level of correlation between the microarray and QRT-PCR results. For statistical verification, the computed Pearson product-moment correlation coefficient was calculated; a significant correlation between the QRT-PCR and microarray data sets was found ($r = 0.900$; $P < 0.0001$). For the eight non-significantly regulated genes there was also a strong correlation ($r = 0.960$; $P < 0.001$) between calculated fold change and *P* values from microarray and QRT-PCR analysis (data not shown). Overall, our correlation analysis confirms the validity of the observed differential expression from the microarray analysis and provides confidence in the detailed normalisation and statistical procedures undertaken in this study.

Expression of glutathione *S*-transferase protein

Using SDS-PAGE and Western blotting, we identified the rGSTA5 protein (25–28 kDa) in the hepatic cytosol from both saline- (lanes 1 and 2) and oxycodone- (lanes 3 and 4) treated rats, and a GST isoform with similar electrophoretic properties (lanes 5 and 6), likely GSTA3, in standard murine S9 fraction (Figure 1). Electrophoretic mobility of the observed GST isozyme proteins is similar to previous findings summarised by Hayes and Pulford.[16] Optical density calculations (mean ± SD, $n = 4$) of rGSTA5 bands normalised to β-actin control for saline and oxycodone

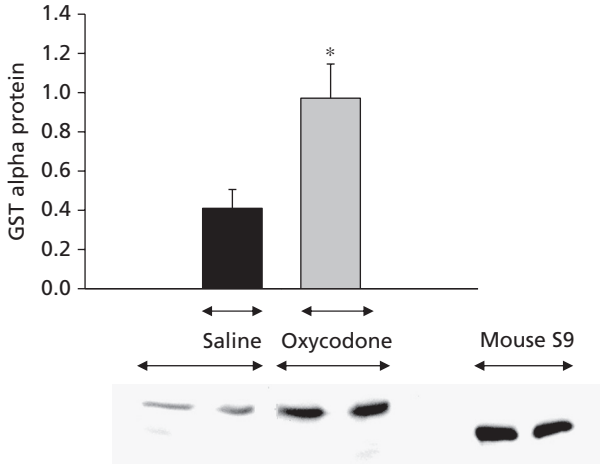

Figure 1 Effects of repeated oxycodone treatment on the protein expression of hepatically expressed rGSTA5. Optical density computations (histogram) in arbitrary units were normalised to β-actin control, indicating a significant increase of rGSTA5 in oxycodone-treated rats. Representative images, including standard murine fraction (S9), from Western blotting are shown for visual comparison. Bars represent means ± SD (n = 4) of normalised band densities. $^{*}P$ = 0.03 vs saline control.

groups (Figure 1) were 0.410 ± 0.100 and 0.970 ± 0.170, respectively (P = 0.008). Thus, we observed a significant elevation (2.5-fold change; 140%) in rGSTA5 protein levels (arbitrary units) in oxycodone-treated rats.

Expression of CYP3A2 protein

SDS-PAGE and Western blotting analyses also identified CYP3A2 protein (56–58 kDa) in liver microsomes from saline- (lanes 1–3) and oxycodone-treated rats (lanes 4–6) and standard rat liver microsomes (lanes 7 and 8) (Figure 2).

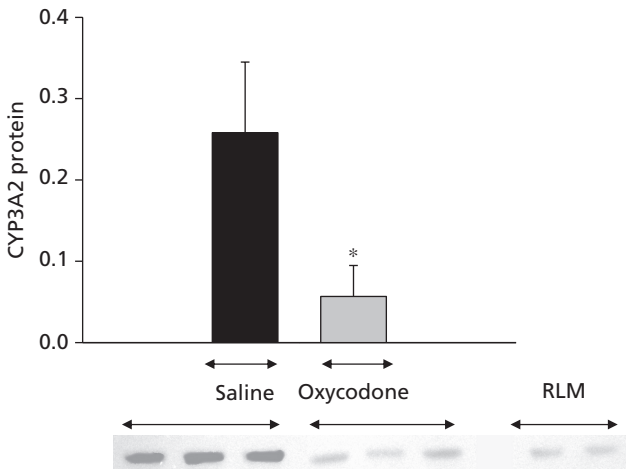

Figure 2 Effects of repeated oxycodone treatment on the protein expression of hepatically expressed CYP3A2. Optical density computations (histogram) in arbitrary units were normalised to β-actin control, indicating a significant repression of CYP3A2 in oxycodone-treated rats. Representative images, including standard rat liver microsomes (RLM), from Western blotting are included for visual comparison. Bars show means ± SD (n = 6) of normalised band densities. $^{*}P$ = 0.02 vs saline control.

Electrophoretic mobility of the observed CYP3A2 is similar to the antibody manufacturer's datasheet (Abcam) and previous isolation and identification of this isoenzyme.[29] Optical density calculations (mean ± SD, n = 6) of CYP3A2 bands normalised to β-actin control for saline and oxycodone groups (Figure 2) were 0.260 ± 0.09 and 0.06 ± 0.04, respectively (P = 0.03). Hence, we observed a significant repression (5.0 fold change; 80% decrease) in CYP3A2 protein levels (arbitrary units) in oxycodone-treated rats.

Apparent glutathione *S*-transferase activity

To assess apparent cytosolic GST activity, the apparent rate of formation of the CDNB-SG adduct was measured in the cytosolic fraction of oxycodone- and saline-treated rat liver using UV spectroscopy at 340 nm. At CDNB concentrations of 320–1000 μmol/l (n = 4 or 5) we observed a significantly greater mean apparent rate of conjugation in the oxycodone-treated liver ($P < 0.05$; Table 3). Percent increases in velocity (Bonferroni's t-test P value) for the oxycodone group at CDNB concentrations of 320, 640, 820 and 1000 μmol/l were 14% (0.003), 20% (< 0.001), 12% (< 0.001), and 10% (0.003), respectively.

Apparent selenium-independent glutathione peroxidase activity

Several GST alpha isoenzymes, including rGSTA5, exhibit GSH peroxide activity and catalyse the reduction of organic hydroperoxides to their corresponding alcohols.[16] This reaction can be measured indirectly by UV spectroscopy via NADPH depletion, since NADPH is an important cofactor consumed in the regeneration of GSH through the reduction of the GSH disulfide.[31] We observed a significantly greater mean apparent rate of CuOOH reduction ($P <$ 0.05; Table 3) in oxycodone-treated rat liver compared with saline-treated liver. This equated to percent changes

Table 3 Spectrophotometric measurement of apparent cytosolic glutathione *S*-transferase activity in the hepatic cytosol of male Sprague-Dawley rats following repeated daily administration of oxycodone 15mg/kg i.p. or saline control

CDNB concn (μmol/l)	Formation of CDNB-SG adduct	
	Saline group	Oxycodone group*
320	410 ± 10	490 ± 10†
640	500 ± 10	600 ± 20‡
820	615 ± 15	690 ± 10‡
1000	660 ± 25	720 ± 10†
CuOOH concn (μmol/l)	**Selenium-independent GSH peroxidase activity**	
	Saline group	Oxycodone group*
30	240 ± 5	295 ± 20*
60	375 ± 10	430 ± 20*
150	420 ± 15	520 ± 30‡
600	480 ± 25	610 ± 30‡

CDNB, 1-chloro-2,4 dinitrobenzene; CuOOH, cumene hydroperoxide; GSH or SG, glutathione. Activity units of CDNB-SG formation and NAPDH oxidation are expressed as nmol product/mg protein per min. Values are means ± SEM of 4 or 5 separate determinations, $^{*}P < 0.05$, $^{\dagger}p < 0.01$ and $^{\ddagger}P < 0.001$.

(Bonferroni's *t*-test *P* value) of 23% (0.042), 15% (0.040), 24% (0.001), and 27% (< 0.001) at CuOOH concentrations of 30, 60, 150 and 600 μmol/l, respectively. Incubations containing all components except CuOOH showed a negligible rate of NADPH depletion (data not shown), confirming that there were no additional enzymes in the isolated hepatic cytosol that contributed to the observed reduction reaction.

Testosterone hydroxylase activity

Testosterone hydroxylation is catalysed by a diverse group of CYPs with a large degree of regio- and stereo-selectivity.[29,32] CYP3A2 (formerly PB-1[33]) displays high testosterone 6β-hydroxylation activity.[33,34] Hence, we selected a previously validated TLC assay to measure 6β-hydroxylation of testosterone in hepatic microsomes from the two treatment groups. The calculated mean ± SD rate (pmol/min per mg protein) of 6β-hydroxylation in oxycodone- and saline-treated microsomes was 200 ± 15 and 650 ± 50, respectively, indicating a 3.3-fold (70%) reduction in the oxycodone-treated sample (P = 0.02).

Discussion

Oxycodone is an opioid receptor agonist, exhibiting potent analgesic and euphoretic properties, and has been used clinically worldwide since the early 1900s.[35] Previously we reported that oxycodone significantly up-regulates the major drug efflux transporter P-gp (ABCB1) in various tissues, including the liver and brain.[14] A subsequent in-vivo study confirmed that oxycodone treatment altered the tissue disposition of the P-gp substrate paclitaxel. Recently, we showed that oxycodone modulated a diverse array of genes in rat brain tissue, including the drug efflux transporter Abcg-2 (BCRP).[15] Moreover, oxycodone impeded the brain accumulation of the Abcg2 substrate mitoxantrone, resulting in an undesirable drug–drug interaction.[15] An initial pilot microarray study conducted in our laboratories revealed that oxycodone altered the gene expression of several hundred unique genes in the liver tissue of oxycodone-treated rats (A. Myers and N. Eddington, unpublished data). Among the vast array of changed genes in this preliminary study, we noted several DMEs. The current study represents a more thorough and focused study into oxycodone-related changes in hepatic gene expression and function of DMEs in male Sprague-Dawley rats.

In the current study rats were administered oxycodone hydrochloride at a dosage of 15 mg/kg every 12 h for 8 days. This dose was carefully selected and pharmacologically relevant, as described in detail elsewhere.[15,19,20,36] In addition, twice-daily dosing of oxycodone is commonly reported in the literature,[14,37] since oxycodone has an elimination half-life of 3.0 ± 0.5 h and five half-lives (> 15 h) are required for ~95% of the drug to be eliminated.[38] As such, dosing every 12 h ensures that enough oxycodone is administered prior to complete elimination from the rats' bodies.

Following repeated treatment of oxycodone, ten DMEs were identified as either significantly down- or up-regulated on our microarray profile and subsequent QRT-PCR validation analysis (Table 1). We selected one down-regulated gene – CYP3A2 – for further protein expression and functional activity analyses because of its key role in metabolising a vast array of endogenous and exogenous compounds.[18] CYP3A2 preferentially catalyses the oxidation of testosterone at the 6β position, although it does exhibit minor 2β and 15β hydroxylase activity.[33,39] In humans, CYP3A enzymes, chiefly CYP3A4, are the most abundantly expressed CYP enzymes and exhibit a broad substrate specificity.[40] Moreover, human CYP3A4 shares 72% sequence identity with rat CYP3A2, indicating possible shared substrate similarities.[41]

Microarray analysis revealed a down-regulation (7.0-fold decrease) of CYP3A2 that was confirmed by QRT-PCR (6.0-fold decrease). Immunoblotting of CYP3A2 (Figure 2) found a corresponding decrease in protein expression (5.0-fold decrease). In addition, our CYP3A2-specific testosterone hydroxlyase activity assay found a significantly decreased rate of formation of the 6β hydroxy metabolite. All in all, the microarray, QRT-PCR, Western blotting and functional assay confirm the down-regulation of CYP3A2 in oxycodone-treated rats.

Interestingly, two other DMEs also involved in testosterone metabolism, CYP17A1 and CYP2C13, were differentially expressed on microarray and QRT-PCR. CYP17A1, up-regulated over 10.0-fold on microarray and QRT-PCR, catalyses the stereospecific hydroxylation of pregnenolone, leading to precursors of the glucocorticoid cortisol, and also plays a key role in androgen biosynthesis.[42] Rat CYP2C13 (P-450g), down-regulated by approximately 3.5 fold on microarray and QRT-PCR, is a highly polymorphic male-specific isozyme that catalyses the hydroxylation of testosterone,[43] androstenedione and progesterone.[44] Thus, the changes in protein expression and functionality of CYP3A2 by oxycodone coupled to potential changes in protein expression and enzymatic acitivity of CYP17A1 and CYP2C13 suggest that chronic oxycodone treatment may significantly alter endogenous testosterone metabolism. For instance, since both down-regulated CYP2C13 and CYP3A2 metabolise testosterone to 6β-hydroxytestosterone, oxycodone treatment may boost circulating endogenous testosterone levels. Also the pronounced up-regulation of CYP17A1 may accelerate the metabolic pathway that produces androgens and cortisol. In fact, Poyhia *et al.*[45] found a significant dose-related increase in short-term cortisol levels in oxycodone-treated patients undergoing anaesthesia. Taken together, our findings clearly warrant future in-vivo studies in males to examine the relationship between oxycodone treatment and circulating androgen levels.

Our microarray and QRT-PCR results also revealed a significant up-regulation of rGSTA5 (Table 1), a member of the large class of GSTs that primarily catalyse the nucleophilic attack of GSH on the electrophilic centre of a variety of compounds, in most cases forming less-toxic, more water-soluble GSH conjugates.[16] Since rGSTA5 is an important enzyme that detoxifies various clinically relevant drug substrates,[16,17] we further characterised its up-regulation in the hepatic cytosol of oxycodone-treated rats. Immunoblotting showed a significantly increased level of rGSTA5 protein in oxycodone-treated rats (Figure 1). Two

separate functional studies were also performed to determine if the increases in mRNA and protein levels led to enhanced cytosolic GST enzymatic activity. Initially, we measured the apparent rate of CDNB conjugation: the oxycodone-treated group showed a significantly greater apparent rate of CDNG-SG formation (Table 3). As previously mentioned, GSTs also contain selenium-independent GSH peroxidase activity that is important in cellular defence against a wide variety of organic hydroperoxides.[46] Enzymatic reduction of CuOOH appears to be confined to mostly GST alpha isozymes.[16] Since our microarray analysis revealed no significant changes in RNA levels of related GST isoenzymes rGSTA3 and rGSTA1, this assay should be more specific to our target isozyme. In fact, our results show an elevated rate in CuOOH reduction in the hepatic cytosol of oxycodone-treated rats (Table 3).

Overall, the fold changes of rGSTA5 RNA levels, protein expression and functional activity observed in this study were 6.5, 2.5 and 1.5, respectively. Similar to our findings, modulation of rGSTA5 RNA by various xenobiotics was reported to show a disproportionate correlation with cytosolic protein expression and GST activity.[25,27,47,48] In addition, the modest but statistically significant changes in GST activity observed in our assays may result in part from pooling of the liver samples, which leads to a more conservative measure.

Taken together, both GST functional studies indicate that there is a significant increase in GST (mainly rGSTA5) activity in the liver tissue of oxycodone-treated rats. The likely human orthologue of rGSTA5 is hGSTA1 or hGSTA3. For example, our sequence comparison analysis using BLAST searches revealed that the amino acid sequence of rGSTA5 (NP_113697) is closely similar to hGSTA1 (NP_665803) and hGSTA3 (NP_000838) – by 87 and 88%, respectively. Human GSTA1 is widely expressed in the liver, whereas hGSTA3 is selectively expressed in steroidogenic tissues.[49] Human GSTA1-1 (the homodimer of two GST α1 subunits) catalyses the GSH conjugation (leading to inactivation) of numerous clinically used nitrogen mustard anti-cancer agents, including chlorambucil, melphalan, cyclophosphamide and thiotepa.[17] In fact, GSTA1-1 was 40–50-fold more proficient than GSTP1-1 in conjugating chlorambucil,[50] and was the only GST isozyme found to catalyse the formation of mono- and diglutathionyl adducts of phosphoramide mustard, a hydrolysis product of 4-hydroxy cyclophosphamide.[51] Thus, the induction of rGSTA5 by oxycodone may increase the risk of certain drug–drug interactions. For example, the co-administration of oxycodone and nitrogen mustard compounds in cancer patients may result in an unwanted reduction in cytotoxic efficacy In addition, CYP3A activates the anti-cancer compounds cyclophosphamide and ifosphamide,[52] further complicating the pharmacotherapy in cancer patients treated long term with oxycodone and one of these agents.

Conclusions

Our study is the first report, to our knowledge, to show the effects of chronic oxycodone administration in male Sprague-Dawley rats on the gene expression of various DMEs, including the testosterone hydroxylase CYP3A2 and dextoxicant rGSTA5. Microarray, QRT-PCR, Western blotting and functional studies all indicate a significant decrease in CYP3A2 and concomitant significant increase in rGSTA5 mRNA, protein expression and functional activity. The potential clinical ramifications of our novel findings are intriguing, and justify future in-vivo pharmacokinetic/pharmacodynamic studies.

Declarations

Conflict of interest

The Author(s) declare(s) that they have no conflicts of interest to disclose.

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