Isoform-dependent changes in cytochrome P450-mediated drug metabolism after portal vein ligation in rat

Tibor Kovács[1], Máté Déri[2], András Fülöp[1]; Tímea Pálházy[1]; Edit Háfra[2], Dávid Sirok[2], Ferenc Ádám Kiss[2], Gábor Lotz[3], Attila Szijártó[1], Katalin Monostory[2*]

[1] Hepato-Pancreatico-Biliary Surgery Research Center Hungary, 1st Department of Surgery, Semmelweis University, Budapest, Hungary

[2] Institute of Enzymology, Research Centre for Natural Sciences, Hungarian Academy of Sciences, Budapest, Hungary

[3] 2nd Department of Pathology, Semmelweis University, Budapest

Short title: Drug metabolism after portal vein ligation

*** Corresponding author:**

Katalin Monostory Ph.D.

Address: H-1117, Magyar tudósok körútja 2, Budapest, Hungary

Mailing address: Katalin Monostory, H-1519 Budapest, Postbox: 286

Phone: +36 1 382 6747

E-mail: monostory.katalin@ttk.mta.hu

Abstract

Background. Surgical removal of complicated liver tumors may be realized in two stages via selective portal vein ligation, inducing the atrophy of portally ligated and the compensatory hypertrophy of non-ligated liver lobes. Unlike morphological changes, functional aspects, such as hepatic cytochrome P450 (CYP)-mediated drug metabolism remain vaguely understood, despite its critical role in both drug biotransformation and hepatic functional analysis. Our goal was the multilevel characterization of hepatic CYP-mediated drug metabolism after portal vein ligation in rat.

Methods. Male Wistar rats (n=24, 210-230g) were analyzed either untreated ('controls'; n=4) or 24/48/72/168/336h (n=4 each) following portal vein ligation affecting ~80% liver parenchyma. Besides the weights of ligated and non-ligated lobes, pentobarbital (30 mg/kg)-induced sleeping time, CYP1A(2), CYP2B(1/2), CYP2C(6/11/13), CYP3A(1) enzyme activities and corresponding isoform mRNA expressions, as well as CYP3A1 protein expression were determined by *in vivo* sleeping test, CYP isoform selective assays, polymerase chain reaction and immunohistochemistry, respectively.

Results. Portal vein ligation triggered atrophy in ligated lobes and hypertrophy non-ligated lobes. Sleeping time was transiently elevated (P=0.0451). After an initial rise, CYP1A, CYP2B and CYP3A enzyme activities dropped until 72h, followed by a potent increase only in the non-ligated lobes; paralleled by an early (24-48h) transcriptional activation only in non-ligated lobes. CYP2C enzyme activities and mRNA levels were bilaterally rapidly decreased, showing but a late reconvergence only in non-ligated lobes. CYP3A1 immunohistochemistry indicated substantial differences in positivity in the early period.

Conclusions. Beyond the atrophy-hypertrophy complex, portal vein ligation generated a transient suppression of global and regional drug metabolism, re-established by an adaptive, CYP isoform-dependent transcriptional response of the non-ligated lobes.

Keywords: cytochrome P450, drug metabolism, liver function, portal vein ligation, rat

Introduction

Liver malignancies remain a major medical problem, being the sixth most frequent tumorous disease worldwide as well as the second in cancer-related deaths [1]. The first-choice treatment is surgical resection to achieve complete tumor removal [2]; performable however in only 10-30% of patients, largely due to an insufficient amount of the future liver remnant [2,3], and an associated increase in liver failure-related morbidity and mortality [4]. For the treatment of patients with low future liver remnant, portal vein occlusive (PVO) strategies may provide an option [2]. Accordingly, surgical ligation (portal vein ligation – PVL) [5] or embolization of portal vein branches of the tumorous lobes (portal vein embolization) [6] induce the atrophy of tumor-bearing segments and a compensatory hypertrophy of the contralateral, (presumably) healthy liver, to ultimately enable liver resection in a second stage [7]. Optimal timing is crucial for the *Scylla and Charybdis* scenario of premature resection induced hepatic dysfunction and imminent tumor progression [7]. Currently, the date of resection is determined using CT volumetry involving the preoperative delineation of the future liver remnant, with consensus threshold values being 25-30% and 40-50% of the tumor-free total liver volume, in healthy or compromised parenchyma, respectively [2-4,7]. However, recent findings pointed out that liver volume may inaccurately reflect liver function, only to highlight the special importance of hepatic functional analysis [8,9]. Therein many of the vast variety of established liver function tests rely on the quantification of hepatic capacity to convert previously administered chemicals, which dominantly depends on the cytochrome P450 enzymes (CYPs) of hepatic drug metabolism [4,9]. CYPs are a superfamily of hemethiolate monooxygenases, located in the smooth endoplasmic reticulum of liver cells and many extrahepatic tissues (e.g. intestine, kidney, lung and brain) and are classified into families and subfamilies according to similarities in their amino acid sequence [10,11]. CYPs play a central role in the metabolism of a large variety of exogenous and endogenous

compounds, and catalyze the phase I oxidative reactions of many drugs, nutrients, environmental pollutants and endogenous substances (steroid hormones, prostaglandins, bile and fatty acids) [11], with CYP 1, 2 and 3 families responsible for the biotransformation of drugs [12,13]. While CYPs generally possess wide substrate specificity, their activity is influenced by both genetic and non-genetic factors (nutrition, co-medication, age, hormonal status, liver function), resulting in substantial intra- and interindividual differences in drug metabolism [14,15]. Beyond these, CYP expressions and activities are also influenced by different pathophysiological conditions, such as infection, inflammation, cancer, or even PVO [16,17]. Therefore, any alterations in drug metabolism are critically important during PVO procedures. For the core significance in liver function and functional analysis, drug metabolism confers key information on functional changes following PVO. Moreover, changes in CYP levels or activities may well result in severe derailment of chronic drug regimens, potentially culminating in undesired side effects or therapeutic failures [13]. Whereas, profiling of CYP expressions and activities may reflect on hepatic drug metabolism [18]. New insights could lead to not only an improved knowledge in liver function testing and the better itineration of resection, but also an enhanced utilization and personalization of perioperative drug doses. Consequently, the aim of our study was the investigation of the transient changes of functional alterations in CYP-mediated drug metabolism following PVL in an experimental rat model involving healthy liver parenchyma.

Materials and methods

Animals and ethics

Experimental design was harmonized with the guidelines of the National Institutes of Health and the 40/2013 (II.14.) Act of the Hungarian Government about animal experiments, as well as actual ARRIVE Guidelines [19]. The experiment was mutually approved by local and institutional ethical boards (Scientific and Ethical Board of Animal Experimentation of the

National Department of Food-chain Safety; the Animal Welfare Ward of the Food-chain Safety, Plant- and Land Protection Division of the Pest County Government Office; and the Animal Welfare Committee of the Semmelweis University (license numbers: PE/EA/2893-6/2016; PEI/001/313-4/2014). Male Wistar rats, aged 7 weeks, weighing 210-230 g (Central Animal Facility, Semmelweis University, Budapest, Hungary) were used in the experiment.

Experimental design and surgical procedure

Following the induction of general anesthesia [intraperitoneal (ip.) injection of 75 mg/body weight (bw) kilograms (kg) ketamine and 7.5 mg/bw kg xylazine in 1.5 ml saline], rats underwent median laparotomy and PVL affecting approximately 80% of total liver parenchyma (n=20), as previously described by our workgroup [20,21]. After abdominal saline lavage, antibiotic (10 mg/bw kg metronidazol ip.) and postoperative analgesic (1 mg/bw kg nalbuphine subcutaneously, repeated once 24h later) treatment and double-layered abdominal suturing, rats were allowed to rest in their cages. Following different postoperative survival times of 24 hours (h)/48h/72h/168h/336h, rats (n=4 each), as well as another group of rats, spared the operative procedure to be used as controls (n=4), were all subjected to the pentobarbital sleeping test. Its finalization was followed by the repeated, previously described ip. anesthetic injection to allow sacrificing of the animal with right ventricular exsanguination for the harvest of liver samples (Figure 1).

Pentobarbital sleeping test

Pentobarbital induced sleeping time was measured to evaluate *in vivo* hepatic CYP activity after ip. injection of pentobarbital (30 mg/bw kg in 1 ml saline). The easing rat was placed lying on its back, and the start of the pentobarbital-induced sleep was defined as the moment when the instinctive reaction of the rat of turning to one side (referred to as the ‘righting reflex’) was completely lost, whereas the end was determined by the spontaneous

reappearance of the righting reflex, with the total sleeping interval specified as ‘sleeping time’ [22].

Liver lobe weights

Secondary to exsanguination, the liver lobes associated with either the ligated [ligated lobes (LL)] or intact [(non-ligated lobes (NLL)] portal vein branches were both measured with a laboratory scale (Mettler Toledo AG 245, Mettler-Toledo LLC, Columbus, OH, confidence: 0.01 mg/0.1 mg), and their individual, as well as combined wet weights (total liver weight) were expressed as per bw, as well as total liver weight.

Preparation of liver microsomes and CYP enzyme activity assays

Liver tissues from both the LL and NLL were homogenized in 0.1 M Tris-HCl buffer (pH 7.4) containing 1 mM EDTA and 154 mM KCl (Reanal Finechemicals Co., Budapest, Hungary). Hepatic microsomal fractions were prepared by the method of differential centrifugation [23] and were stored at -80º C until further analysis. The protein content of the microsomes was determined by the method of Lowry et al. [24], with bovine serum albumin as the standard (Sigma-Aldrich GmbH, Deisenhofen, Germany). Subsequently, CYP-selective enzyme activities were determined by previously published methods, involving ethoxyresorufin *O*-deethylation for CYP1As [25], pentoxyresorufin *O*-dealkylation for CYP2Bs [25], tolbutamide 4-hydroxylation for CYP2Cs [26], and midazolam 1’- and 4-hydroxylation for CYP3As, respectively [27]. The incubation mixture contained a NADPH-generating system (1 mM NADPH, 10 mM glucose 6-phosphate, 5 mM $MgCl_2$, and 2 international units/ml glucose 6-phosphate dehydrogenase), rat liver microsomes, and the respective CYP isoform-specific substrate (ethoxyresorufin, pentoxyresorufin, tolbutamide or midazolam for CYP1As, CYP2Bs, CYP2Cs and CYP3As, respectively). Enzyme reactions were terminated by either ice-cold methanol or acetonitrile. High performance liquid

chromatographic analyses were performed while adhering to published methods [26,27]. The fluorescence of resorufin was quantified at 550 nm excitation and at 589 nm emission wavelengths [25]. Finally, beyond the determination of 'intrinsic' microsomal CYP isoform activities, expressed as [pmol metabolite/(mg microsomal protein*min)], the total CYP isoform activities of the LL and the NLL were also extrapolated after scaling with the corresponding weights of the LL or NLL, and displayed in relative units (per cent of control).

RNA isolation and quantitative real-time polymerase chain reaction (RT-PCR)

Total RNA was isolated from liver samples (TRI reagent, Molecular Research Center Inc., OH) according to the manufacturer's instructions. The purity and the concentration of the RNA samples were determined spectrophotometrically (NanoDrop 1000, Thermo Fisher Scientific Inc., Waltham, MA). RNA (3 µg) was reverse-transcribed into single-stranded cDNA (Maxima First Strand cDNA Synthesis Kit, Thermo Fisher Scientific Inc.), and RT-PCR was performed (Maxima SYBR Green qPCR Master Mix, Thermo Fisher Scientific Inc.) using the respective primers for CYP isoforms: CYP1A2, CYP2B1, CYP2B2, CYP3A1, CYP2C6, CYP2C11, CYP2C13 (Table 1). The quantity of the target mRNA relative to that of the housekeeping gene hypoxanthine phosphoribosyltransferase (HPRT) was determined [28].

Histopathological analysis

Liver samples were excised from identical anatomical parts of the LL and the NLL, fixed in 4% neutral-buffered formaldehyde, and embedded in paraffin. Sections of 3-5 µm were cut, deparaffinized (EZ Prep 10x, F. Hoffmann-La Roche AG Konzern, Basel, Switzerland) and rehydrated. Antigen retrieval was performed for 30 min with a prediluted solution (Cell Conditioning 1, Ventana Medical Systems, F. Hoffmann-La Roche AG Konzern) designated for automated immunohistochemical slide stainers. A standard immunohistochemical reaction was performed in an automated immunohistochemical reaction slide staining system

(BenchMark ULTRA, Ventana Medical Systems, F. Hoffmann-La Roche AG Konzern) with a respective detection kit (ultraView Universal DAB Detection Kit, F. Hoffmann-La Roche AG Konzern) involving a standard horseradish peroxidase-diaminobenzidine reaction. Rabbit polyclonal primary antibodies against CYP3A1 (BML-CR3310-0025; Enzo Life Sciences Inc., Farmingdale, NY) were applied at 42°C for 32 min in a dilution of 1:1000. Manufacturer instructions were always followed. Sections were counterstained with hematoxylin and scanned to produce digital material.

Statistical analysis

Data were expressed as mean ± standard deviation. Statistical analysis of parametric data involved analysis of variance test with Bonferroni's *post hoc* test. Statistical significance was declared in case of *P*-values equaling or below 0.05. GraphPad Prism 6 (GraphPad Software Inc., La Jolla, CA) was used for analyses and visualization.

Results

Pentobarbital sleeping test

Sleeping time gradually increased after PVL with a significantly elevated peak at 72h (P=0.0451), and thereafter normalized to control levels (Figure 2). However, sleep latency (the time for complete loss of righting reflex after pentobarbital injection) at each time point was similar to that of the control group (data not shown).

Liver lobe weights

Following PVL, the weight of the LL, constituting 76±1.83% of total liver weight and 3.77±0.27% of bw in controls, progressively decreased to only 15.25±7.18% of total liver weight and 0.63±0.33% of bw by 336h (P<0.0001). Meanwhile, NLL weights significantly increased from the control values of 23.25±1.71 % of total liver weight and 1.17± 0.05 % of bw to 84.25±6.4% of total liver weight and 3.37±0.29 % of bw by the end of the experiment

(P<0.0001). A slight, but significant decrease in total liver weight was observed 24h after PVL; however, total liver weight remained essentially unchanged thereafter (Figure 3).

In vitro CYP enzyme activity assays

The 'intrinsic' microsomal CYP activities (pmol / mg protein*min) were assessed initially (Figures 4-7). Enzyme activities of CYP1A and CYP3A (measured both by midazolam 1'- and 4-hydroxylation) displayed very similar characteristics (Figures 4a, 7a, 7c). 24h after PVL, enzyme activities were significantly increased in the NLL, after which the activities of both lobes gradually decreased until 72h. Thereafter however, the enzyme activities diverged. In the NLL, a significant increase in ethoxyresorufin *O*-deethylation as well as in midazolam 1'- and 4-hydroxylation was observed at 168h compared to control values, followed by convergence to baseline by 336h. Meanwhile, enzyme activities in LL, despite a temporary insignificant gain, exhibited a downward tendency beyond 48h. Consequently, a discrepancy developed between enzyme activities of LL and NLL, which resulted in a statistically significant difference as early as 24-48h, and became the most prominent at the late time points of 168-336h. CYP2B enzyme activities showed some similarities to that of CYP1A and CYP3A; however, both the late rise of CYP2B activity in NLL, and concomitantly, the discrepancy of LL and NLL activities were moderate and only tendentious (Figure 5a). CYP2C activities displayed completely different features (Figure 6a). Tolbutamide 4-hydroxylation activity of both the LL and the NLL underwent a sharp and significant decrease immediately post-PVL. However, after 168-336h, enzyme activity in the NLL showed a late convergence to the baseline, while the LL remained permanently depressed, which also resulted in a gaping discrepancy and a significant difference of CYP2C enzyme activities between LL and NLL at the late time points of 168-336h. Changes of total CYP activities extrapolated from 'intrinsic' activities by scaling with the weight of LL or NLL were generally very similar to the corresponding 'intrinsic' microsomal CYP activities, with

matching curve characteristics (Figures 4b, 5b, 6b, 7b, 7d). However, the ‘up-scaling’ with LL and NLL weights further amplified the results observed in terms of ‘intrinsic’ microsomal activities primarily in the later stages. Thereby, while the lowered activity of the LL further sank, the progressively increased activity of the NLL became more prominent due to the shift of lobe weights, resulting in significantly elevated total NLL CYP1A, CYP2C, and CYP3A function even at 336h, as well as a greater interlobar discrepancy, also in terms of CYP2B. Subsequent to the surge in total CYP activity of the NLL, the combined total CYP1A and CYP3A activities of the liver were also significantly elevated at 168h compared to control values. Whereas the redistribution of total liver CYP function was observable in terms of all isoforms, reflected in a rise from the 28±2% control functional contribution of the NLL to the total extrapolated CYP function of the liver to 96%±2% by 336h ($P<0.0001$).

Transcriptional expression of CYP enzymes

The mRNA levels of CYP2B1, CYP2B2, CYP3A1 reflected generally similar changes (Figures 5c, 5d, 7e). Here, mRNA levels of the NLL showed an early, steep and significant increase in the 24-48h period, followed by a rapid redistribution to baseline levels. Meanwhile, mRNA levels in the LL showed either only a modest increment (CYP2B1 and CYP2B2) or were unchanged (CYP3A1). Hence, a significant difference was verified between LL and NLL mRNA expression levels, confined to the early postoperative period of 24-48h, peaking at 48h. While a gap of LL and NLL mRNA expression levels was also present in case of CYP1A2 (Figure 4c), instead of only an early localization, it was observable throughout the whole postoperative period from 24h to 336h, which was generated by a dramatic, immediate and definite reduction of CYP1A2 mRNA expression in the LL, in parallel to an upheld (and at 48h, tendentiously increased) mRNA expression of the NLL. Again, CYP2C6, CYP2C11, CYP2C13 mRNA concentrations displayed different characteristics (Figure 6c-e). While the mRNA levels of the LL were always significantly and

permanently depressed, CYP2C mRNA expression in the NLL was either maintained (CYP2C6), or showed only a temporary decrement with later re-convergence either bordering (CYP2C13), or even reaching the baseline levels (CYP2C11). As a result, a significantly higher mRNA expression in the NLL was once again displayed dominantly in the later stages of 168-336h, but was also present at 48h in terms of CYP2C6 and CYP2C13.

Histopathological analysis of CYP3A1 protein

Representative images of the CYP3A1 immunohistochemical reaction are shown at various termination timepoints (Figure 8). CYP3A1 positivity of the LL decreased after PVL and remained permanently lower. While positivity in the NLL was also lower as compared to controls at 168h, a maintained expression between 24-72h resulted in a difference of CYP3A1 positivity between LL and NLL in the early postoperative period.

Discussion

Future liver remnant enlargement via PVO remains the sole curative option for a wide range of patients with liver malignancies [3,7,29]. During the induced liver regeneration process, the precise alterations of hepatic drug metabolism are still incompletely understood, despite its key role in both hepatic function and the metabolism of endogenous and exogenous compounds [30]. Our primary objective was the temporal and regional, multi-level characterization of hepatic drug metabolism and its inherent CYP enzymes during PVL induced liver regeneration in rat. The present study found an early, transient deterioration of the global drug metabolizing capacity, which was echoed by the contemporary individual functions of both ligated and non-ligated lobes. We verified a solid, disparate difference between the drug metabolism of the LL and NLL. As a result of an adaptive response of active transcriptional upregulation, drug metabolism in the NLL was increased and re-established, meanwhile, permanently depressed in the LL due to the lack of such adaption.

Hepatic drug metabolism became overwhelmingly redistributed, depending nearly exclusively on the NLL. Our findings also proved the isoform-dependence of CYP enzyme activity and transcription alterations following PVL in rat.

PVL induces a chain of events, ultimately culminating in a very special form of liver regeneration [5,6]. Eventually, the LL undergoes atrophy and underlying necroapoptotic cell death, meanwhile the NLL displays an intensive cellular proliferation and hypertrophy [21,29], which was repeatedly proven by the literature [2,5,29,30], and previous reports of our workgroup [20,21,31]. In the present study, it was similarly reflected by liver lobe weights; thereby validating the standard application of the PVL model, and allowing further, more sophisticated analysis. Beyond the phenomenon, morphological features following PVO possess key clinical significance, for the timing of stage II resection is consensually determined by CT liver volumetry [2-4,7]. However, (posthepatectomy) liver failure is directly dependent on liver function, but not liver volume, and the two may dramatically differ, according to recent reports [8,9]. Therefore, the significance of functional analysis during PVO procedures is dramatically increasing. A core hepatic function is the ability to convert and eliminate endogenous and exogenous chemical substances, generally referred to as drug metabolism, which directly relies on key CYP enzymes [11-13]. Besides non-genetic factors such as age and hormonal status, CYP activities and expressions are influenced by a variety of environmental and acquired factors such as nutrition, co-medication, infections, inflammation and cancer [15], which may either induce their activities through transcriptional upregulation over days, or oppositely reduce CYP activities through downregulation of transcription or instantaneous posttranslational inactivation [12]. Any changes in CYP enzyme activities could subsequently result in the alterations of drug metabolism, which may lead to adverse drug-related effects of drug intoxication or therapeutic failure [13]. In particular, pathophysiological conditions of the liver, including liver regeneration after partial

hepatectomy [32] or PVO [16], carry the grave danger of reduced hepatic function and CYP activity derangements, which is well-reflected in the CYP-dependence of several quantitative liver function tests [4]. Therefore, an improved understanding of PVO-induced alterations in hepatic drug metabolism and CYP activities holds great clinical potential for the prevention of drug-related complications and the extension of the available knowledge regarding liver function alterations during induced liver regeneration. In our pursuit of the analysis of drug metabolism after PVL, a proximal element was the pentobarbital sleeping test, representing the CYP-mediated metabolism and elimination of pentobarbital. Herein, the overall CYP activity, as well as global drug metabolism was temporarily reduced until 72h after PVL and was retrieved by the end of the second week. In accordance with this, Takemura *et al* [30] and Starkel *et al* [33] also found a transient reduction in hepatic drug metabolism following PVL. Furthermore, on a larger scale, the transitional deterioration of global liver function after PVO, as confirmed by different functional tests, was described both by our and other previous literature reports [20,31,34]. On the other hand, the sleeping test fails to assess the individual functional aspects of the LL and the NLL. Whereas following the first step (i.e. PVO) of two-step liver resections incorporating preoperative liver remnant enlargement by PVO, both the LL and the NLL persist in the body; however, after the interval period and the subsequent second-step resection of the LL, only the NLL is preserved, upon which the metabolic demands of the body rest. Therefore, regional functional analysis, and specifically, the assessment of the NLL function is of utmost importance, for which we embarked on the investigation of drug metabolism as well as the expression and activities of underlying CYP isoenzymes individually in the LL and the NLL. With interspecies differences of sequence, substrate specificity and regulation in mind [12,13], several representative, clinically most relevant CYP isoenzymes were chosen from the CYP1, CYP2 and CYP3 families, which are near-exclusively responsible for the metabolism of drugs and other xenobiotics [11]. CYP1A

enzymes are known for their great interspecies homology; therefore, they provide good ground for comparison between human and rat [11,12]. CYP2B1 and CYP2B2 isoenzymes metabolize anaesthetic drugs, and also provide good comparison between human and rat [11,12]. The CYP3A subfamily is critically important both in humans and rats, for it metabolizes approximately 40% of drugs on the market. They comprise ~30% of CYP content of the human liver, with CYP3A4 as the dominant enzyme. In rat, CYP3As are also essential, even though, they make up a smaller fraction of hepatic CYPs. On the other hand, a number of induction/inhibition differences between human CYP3A4 and its rat correspondent CYP3A1 are known, for which CYP3As provide highly-relevant interspecies comparison, with certain limitations [11-13]. In human, CYP2Cs are of limited significance metabolizing only ~16% of drugs, and constituting just 20% of liver CYP content, whereas in rat, with over 50%, they are the most abundant CYPs contained in the liver with a correspondingly high metabolic relevance. Finally, rat CYP2Cs show great sexual predominance, with CYP2C11 and CYP2C13 as the most prominent male-specific isoforms, whereas CYP2C6 is sex-independent [11-13]. Consequently, in the present study, CYP1A, CYP2B, CYP2C and CYP3A enzyme activities, along with CYP1A2, CYP2B1, CYP2B2, CYP2C6, CYP2C11, CYP2C13, CYP3A1 mRNA levels were measured to better understand alterations in CYP enzyme functions and the underlying transcriptional regulation. The ‘intrinsic’ activities of CYP1A and CYP3A, and to a lesser extent, CYP2B displayed a very similar pattern of an early rise at 24h, followed by a downward tendency up to 72h, after which NLL activities were increased before returning to baseline, meanwhile enzyme activities of the LL remained permanently low. Concomitantly, total lobar activities of the same enzymes were generally matching to that of the ‘intrinsic’ enzyme activities. However, lobe weight-scaling further amplified the discrepancies of the LL and the NLL activities, as well as the potent increase of NLL function at 168-336h, which also led to an increased total liver activity at 168h, and a

massively raised functional share of the NLL of total liver activity, going from the control 28% to 96% at 336h. Whereas, at the mRNA level, sharp contrast was observed between the LL and NLL, for the NLL exhibited a very early transcriptional upregulation of CYP1A2, CYP2B1, CYP2B2 and CYP3A1 at 24-48h, which was lacking in the LL. To interpret these, the increase in CYP enzyme function at 24h is considered to be the consequence of CYP protein stabilization and/or *de novo* protein synthesis in NLL [12]. Meanwhile, CYP2C results were markedly different. Both 'intrinsic' and total lobar activities as well as the mRNA levels of the male-dominant CYP2C11 and CYP2C13 were suddenly sharply reduced in both lobes, and a late re-convalescence of both enzyme functions and mRNA expression to baseline were seen only in the NLL at the late time point of 336h. CYP2C6 expression was also downregulated in LL; however, it did not display any significant fluctuation in NLL. Perhaps the most striking observation is the dramatic difference of lobar responses, as part of which drug metabolism in the LL was permanently deteriorated, whereas re-established in the NLL, which may be explained by the far-reaching effects of PVL. Beyond the seemingly solitary action of portal blood flow stoppage to the LL, PVL induces a chain of events, such as the complete redistribution of the splanchnic circulation (arterialized hypoperfusion of the LL; massive portal hyperperfusion of the NLL) and modification of blood decomposition, by which the NLL receives an increased portion of nutritional agents, growth factors, hormones, and bacterial products [29]. These culminate in the induction of a very special form of liver regeneration characterized by the atrophy-hypertrophy complex [29,35], and diverse pathophysiological changes of structural and microvascular remodeling within the LL and the NLL [20,29], which may ultimately reason the different functional capacities of the LL and the NLL [31,35]. The other important finding that should be addressed is the markedly different pattern of changes regarding the CYP2C enzymes as compared to CYP1A, CYP2B and CYP3A. In the early postoperative period, both lobes exhibited a dramatic reduction in

CYP2C enzyme activity as well as transcription, which may be multifactorial. Earlier reports also confirmed a transiently reduced CYP-dependent drug metabolizing capacity after PVL. Takemura *et al* concluded a lag of functional (CYP3A2, CYP2C11) to morphological regeneration [30], whereas Starkel *et al* reported CYP3A and CYP2E1 functional deterioration in the very early (0.5-8h) postoperative period secondary to surgical stress but not liver regeneration [33]. Regarding the molecular background of CYP depression, both studies agreed on the central role of inflammatory cytokines (IL-1β, IL-6, TNF-α), as well as the upregulation and increased nitric oxide production of inducible nitric oxide synthase [30,33]. During different pathophysiological conditions such as infection, inflammation and cancer, increased levels of circulating proinflammatory cytokines lead to CYP downregulation [17,36] through the repression of nuclear receptors (pregnane X receptor and constitutive androstane receptor) involved in transcriptional regulation of several CYPs (CYP2B, CYP2C, CYP3A) [37,38]. Meanwhile, the same cytokines, as well as nitric oxide also play a central role in the initiation and mediation of liver regeneration after both partial hepatectomy and PVO, when their serum levels are sharply increased secondary to inflammatory activation and hemodynamical changes, respectively [29,35,39]. Therefore, it is highly plausible that increased levels of inflammatory cytokines as well as nitric oxide may be partly responsible for the transient deterioration of CYP function following PVL. As compared to these previous reports, our study is the first to investigate the underlying transcriptional regulation through the analyses of corresponding, specific mRNA expressions parallel to the enzyme activities of a wide range of highly relevant CYP enzymes throughout the full duration of PVL induced liver regeneration. CYP2Cs displayed marked differences in enzyme activity as well as underlying mRNA levels compared to the other subfamilies both in the early and late phase of regeneration. During the latter, the waning effects of cytokines and nitric oxide could not fully account for the discrepancy of CYP2Cs, whereas a possible

explanation might lie in sex hormone-dependent regulation. The expression of CYP2C enzymes, and particularly, the male predominant CYP2C11 and CYP2C13 is regulated by the hypothalamus-hypopituitary-gonadal axis and primarily affected by the actual balance of testosterone/estrogen levels [12]. Besides exogenous chemicals, endogenous disease states are also capable of causing reduced CYP expression or function by interfering with sex hormonal levels [12]. Earlier studies reported that pathophysiological states of the liver (including partial hepatectomy, PVL, bile duct ligation) led to the 'demascularinization' of drug metabolism (decreased expression of male-specific and increased expression of female-specific enzymes) along with lower serum testosterone and increased (gastric parietal cell-generated) estradiol levels, as well as hypothalamic-hypopituitary-gonadal suppression [16,32,40]. Consequently, regarding the present study, an increased presence of female sex hormones may have contributed to the rapid and prolonged decrement of CYP2C enzyme activities and male-specific CYP2C11 and CYP2C13 mRNA expressions, while CYP2C6 expression of the NLL was preserved. In the meantime, the restitution and hyperfunction of sex hormone-independent CYP1A and CYP3A1 activities by 168h and early steep increase of their respective mRNA expressions could indicate a potent adaptive response of the NLL; perhaps an endogenous 'escape' mechanism of the organism to mitigate the functional suppression of CYP2C enzymes. Meanwhile, the LL failed to present such an increment, most likely due to the described severe morpho-structural consequences of PVL. Finally, the representative CYP3A1 immunohistochemical reaction also indicated a preserved early postoperative positivity of CYP3A enzymes in the NLL as compared to the progressively diminished positivity of the LL. Overall, the individual drug metabolism of the NLL seems to be re-established, whereas permanently deteriorated in the LL, confirming disparate tendencies of adaption, and a shift of hepatic drug metabolism towards the NLL. These are in accordance with the latest studies of our workgroup, involving complex liver functional

analyses following PVL [20,31]. Concerning clinical significance, the present results may help characterize liver functional changes during two-step liver resections incorporating future liver remnant enlargement via PVO procedures, translating as the enhanced itineration of the second-step resection. Furthermore, an improved knowledge of liver drug metabolism could help prevent drug complications through the adaption of medication dosage to the actual capacities of hepatic CYPs. This brings about the modern concept of personalized medication and patient-tailored drug regimens, such as the estimation of the drug metabolizing capacity based on the genotyping and expressional patterns of CYPs [18]. The current analysis underlined CYP isoform-dependent responses, which take this personalized approach a step further, by suggesting an even more precisely tailored medication strategy. Therein, drug dosage may be weighed against the contemporary activities of the specifically corresponding CYP isoforms. A noteworthy limitation of our study is the utilization of a 'healthy liver' model, concerning which our goal was the most direct evaluation of the pathophysiological effects of PVO on hepatic drug metabolism. It is only a partial hindrance, since clinically, tumors often develop in parenchymal disease-free livers. Such is the case of metastases, the most frequent liver tumors worldwide [1]. However, (predominantly primary) liver tumours often manifest in disease-affected liver parenchyma (steatohepatitis, fibrosis, cirrhosis, cholestasis, etc.), which may interfere with CYP expression and hepatic drug metabolism. Supporting this, the Heidelberg workgroup found reduced CYP2E1 expression in liver fibrosis, identified with liver stiffness elastography, as well as boosted CYP expression, drug metabolism and subsequently increased, carcinogenic etheno DNA-adducts in (non-fibrotic) alcoholic liver disease [41,42]. Therefore the in-depth evaluation of CYP regulation in liver disease states is also of chief clinical relevance. Further research is imperative for the complex understanding of the role and individual changes of CYP enzymes following PVO procedures in both the absence and presence of parenchymal liver diseases.

Conclusions

PVO and PVL remain key treatment options of complicated liver tumors, during which functional aspects, such as the elimination of endogenous- and exogenous compounds are crucial. Monitoring the alterations in hepatic drug metabolism and underlying CYP enzymes is critically important for better understanding of post-PVO liver function, as well as for the prevention of drug-related complications. Our study verified a transient suppression of global hepatic function, followed by the adaptive response of NLL and the permanent deterioration of the LL resulting in solid interlobar differences. Furthermore, changes were found to be isoform-dependent, with somewhat different expression and activity profiles of CYP1A, CYP2B and CYP3A as compared to CYP2C enzymes. Results may contribute to knowledge regarding liver function as well as personalized medication strategies during induced liver regeneration; however, further investigation is required.

Acknowledgements

The authors thank Katalin Tóth, Erzsébet Kovács Azumahné, Attila Szinvai, and Kristóf Illés for their help in the realization of this experiment. This work was supported by the grants of the National Institute of Research, Development and Innovation [NKFIH/OTKA- K115607; VEKOP-2.3.3-15-2017-00014].

Disclosure Statement

No conflicts of interests to disclose.

References

1 Ferlay J, Soerjomataram I, Dikshit R, Eser S, Mathers C, Rebelo M, Parkin DM, Forman D, Bray F: Cancer incidence and mortality worldwide: sources, methods and major patterns in GLOBOCAN 2012. International journal of cancer 2015;136:E359-E386.

2 Clavien P-A, Emond J, Vauthey JN, Belghiti J, Chari RS, Strasberg SM: Protection of the liver during hepatic surgery. Journal of gastrointestinal surgery 2004;8:313-327.

3 Breitenstein S, Apestegui C, Petrowsky H, Clavien PA: "State of the art" in liver resection and living donor liver transplantation: a worldwide survey of 100 liver centers. World J Surg 2009;33:797-803.
4 Mullin EJ, Metcalfe MS, Maddern GJ: How much liver resection is too much? American journal of surgery 2005;190:87-97.
5 Rous P, Larimore LD: Relation of the portal blood to liver maintenance: a demonstration of liver atrophy conditional on compensation. The Journal of experimental medicine 1920;31:609-632.
6 Kinoshita H, Sakai K, Hirohashi K, Igawa S, Yamasaki O, Kubo S: Preoperative portal vein embolization for hepatocellular carcinoma. World journal of surgery 1986;10:803-808.
7 van Lienden KP, van den Esschert JW, de Graaf W, Bipat S, Lameris JS, van Gulik TM, van Delden OM: Portal vein embolization before liver resection: a systematic review. Cardiovascular and interventional radiology 2013;36:25-34.
8 de Graaf W, van Lienden KP, van den Esschert JW, Bennink RJ, van Gulik TM: Increase in future remnant liver function after preoperative portal vein embolization. British Journal of Surgery 2011;98:825-834.
9 Hoekstra LT, de Graaf W, Nibourg GA, Heger M, Bennink RJ, Stieger B, van Gulik TM: Physiological and biochemical basis of clinical liver function tests: a review. Annals of surgery 2013;257:27-36.
10 Nelson DR: The cytochrome p450 homepage. Human genomics 2009;4:59.
11 Zanger UM, Schwab M: Cytochrome P450 enzymes in drug metabolism: regulation of gene expression, enzyme activities, and impact of genetic variation. Pharmacology & therapeutics 2013;138:103-141.
12 Martignoni M, Groothuis GM, de Kanter R: Species differences between mouse, rat, dog, monkey and human CYP-mediated drug metabolism, inhibition and induction. Expert opinion on drug metabolism & toxicology 2006;2:875-894.
13 Zuber R, Anzenbacherova E, Anzenbacher P: Cytochromes P450 and experimental models of drug metabolism. Journal of cellular and molecular medicine 2002;6:189-198.
14 Solus JF, Arietta BJ, Harris JR, Sexton DP, Steward JQ, McMunn C, Ihrie P, Mehall JM, Edwards TL, Dawson EP: Genetic variation in eleven phase I drug metabolism genes in an ethnically diverse population. Pharmacogenomics 2004;5:895-931.
15 Monostory K, Pascussi J-M: Regulation of Drug-metabolizing Human Cytochrome P450s. Acta Chimica Slovenica 2008;55
16 Farrell GC, Koltai A, Zaluzny L, Murray M: Effects of portal vein ligation on sex hormone metabolism in male rats: Relationship to lowered hepatic cytochrome P 450 levels. Gastroenterology 1986;90:299-305.
17 Slaviero KA, Clarke SJ, Rivory LP: Inflammatory response: an unrecognised source of variability in the pharmacokinetics and pharmacodynamics of cancer chemotherapy. The lancet oncology 2003;4:224-232.
18 Temesvári M, Kóbori L, Paulik J, Sárváry E, Belic A, Monostory K: Estimation of drug-metabolizing capacity by cytochrome P450 genotyping and expression. Journal of Pharmacology and Experimental Therapeutics 2012;341:294-305.
19 Kilkenny C, Browne WJ, Cuthill IC, Emerson M, Altman DG: Improving bioscience research reporting: the ARRIVE guidelines for reporting animal research. PLoS Biol 2010;8:e1000412.
20 Fulop A, Budai A, Czigany Z, Lotz G, Dezso K, Paku S, Harsanyi L, Szijarto A: Alterations in hepatic lobar function in regenerating rat liver. The Journal of surgical research 2015;197:307-317.
21 Fülöp A, Szijártó A, Harsányi L, Budai A, Pekli D, Korsós D, Horváth I, Kovács N, Karlinger K, Máthé D, Szigeti K: Demonstration of Metabolic and Cellular Effects of Portal Vein Ligation Using Multi-Modal PET/MRI Measurements in Healthy Rat Liver. PLoS ONE 2014;9:e90760.
22 Abdolmaleki A, Moghimi A, Ghayour MB, Rassouli MB: Evaluation of neuroprotective, anticonvulsant, sedative and anxiolytic activity of citicoline in rats. European journal of pharmacology 2016;789:275-279.

23 van der Hoeven TA, Coon MJ: Preparation and properties of partially purified cytochrome P-450 and reduced nicotinamide adenine dinucleotide phosphate-cytochrome P-450 reductase from rabbit liver microsomes. Journal of Biological Chemistry 1974;249:6302-6310.
24 Lowry OH, Rosebrough NJ, Farr AL, Randall RJ: Protein measurement with the Folin phenol reagent. Journal of biological chemistry 1951;193:265-275.
25 Burke MD, Thompson S, Elcombe CR, Halpert J, Haaparanta T, Mayer RT: Ethoxy-, pentoxy- and benzyloxyphenoxazones and homologues: a series of substrates to distinguish between different induced cytochromes P-450. Biochemical pharmacology 1985;34:3337-3345.
26 Miners JO, Birkett DJ: [15] Use of tolbutamide as a substrate probe for human hepatic cytochrome P450 2C9. Methods in enzymology 1996;272:139-145.
27 Kronbach T, Mathys D, Umeno M, Gonzalez FJ, Meyer UA: Oxidation of midazolam and triazolam by human liver cytochrome P450IIIA4. Molecular pharmacology 1989;36:89-96.
28 Peinnequin A, Mouret C, Birot O, Alonso A, Mathieu J, Clarençon D, Agay D, Chancerelle Y, Multon E: Rat pro-inflammatory cytokine and cytokine related mRNA quantification by real-time polymerase chain reaction using SYBR green. BMC immunology 2004;5:3.
29 Szijártó A, Fülöp A: Triggered Liver Regeneration: From Experimental Model to Clinical Implications. European Surgical Research 2015;54:148-161.
30 Takemura S, Minamiyama Y, Hirohashi K, Kubo S, Funae Y, Kinoshita H: Recovery of hepatic function determined by cytochrome P450-dependent drug metabolism lags after compensatory hepatic volume changes after portal vein ligation in rats. The Journal of surgical research 2006;134:285-291.
31 Kovács T, Máthé D, Fülöp A, Jemnitz K, Bátai-Konczos A, Veres Z, Török G, Veres DS, Horváth I, Szigeti K, Homolya L, Szijártó A: Functional shift with maintained regenerative potential following portal vein ligation. Scientific Reports 2017;7:18065.
32 Liddle C, Murray M, Farrell GC: Effect of liver regeneration on hepatic cytochrome P450 isozymes and serum sex steroids in the male rat. Gastroenterology 1989;96:864-872.
33 Starkel P, Laurent S, Petit M, Van Den Berge V, Lambotte L, Horsmans Y: Early down-regulation of cytochrome P450 3A and 2E1 in the regenerating rat liver is not related to the loss of liver mass or the process of cellular proliferation. Liver 2000;20:405-410.
34 Hashimoto M, Watanabe G: Changes in functional capacity after portal branch ligation in rats. Surgery today 2001;31:1065-1069.
35 Taub R: Liver regeneration: from myth to mechanism. Nature reviews Molecular cell biology 2004;5:836-847.
36 Aitken AE, Richardson TA, Morgan ET: Regulation of drug-metabolizing enzymes and transporters in inflammation. Annu Rev Pharmacol Toxicol 2006;46:123-149.
37 Pascussi JM, Dvorák Z, Gerbal-Chaloin S, Assenat E, Maurel P, Vilarem MJ: Pathophysiological factors affecting CAR gene expression. Drug metabolism reviews 2003;35:255-268.
38 Pascussi J-M, Gerbal-Chaloin S, Pichard-Garcia L, Daujat M, Fabre J-M, Maurel P, Vilarem M-J: Interleukin-6 negatively regulates the expression of pregnane X receptor and constitutively activated receptor in primary human hepatocytes. Biochemical and biophysical research communications 2000;274:707-713.
39 Schoen JM, Wang HH, Minuk GY, Lautt WW: Shear stress-induced nitric oxide release triggers the liver regeneration cascade. Nitric oxide 2001;5:453-464.
40 Kobayashi H, Yoshida S, Sun Y-J, Shirasawa N, Naito A: Gastric estrogen increases pituitary estrogen receptor α and prolactin mRNAs during the different pathological conditions of the liver. Endocrine 2013;43:170-183.
41 Mueller S, Peccerella T, Qin H, Glassen K, Waldherr R, Flechtenmacher C, Straub BK, Millonig G, Stickel F, Bruckner T: Carcinogenic Etheno DNA Adducts in Alcoholic Liver Disease: Correlation with Cytochrome P-4502E1 and Fibrosis. Alcoholism: Clinical and Experimental Research 2018;42:252-259.
42 Theile D, Haefeli WE, Seitz HK, Millonig G, Weiss J, Mueller S: Association of liver stiffness with hepatic expression of pharmacokinetically important genes in alcoholic liver disease. Alcoholism: Clinical and Experimental Research 2013;37:E17-E22.

Figure legends

Figure 1. Portal vein ligation and experimental setup. Portal vein ligation affecting approxiately 80% rat liver parenchyma was performed according to the previously published method of our workgroup. (a) The common portal branch of the median (ML), left lateral (LLL) and caudate (CL) liver lobes was atraumatically dissected, isolated and surgically ligated with a 6-0 polipropylene suture, in a position (indicated by double arrows) distal to the debranching point of the portal vein tributary of right lateral lobes (RLL). As a result, the ML, LLL and CL liver lobes are deprived of portal vein blood inflow ('ligated lobes' labelled as red), whereas the portal circulation of the RLL reimans intact ('non-ligated lobes' labelled as green). '*' indicates the main trunk of the portal vein. Experimental setup and group order. (b) Five groups of male Wistar rats were initially subjected to the portal vein ligation operation (indicated by black dots), and allowed different survival times of 336, 168, 72, 48 and 24 hours (n=4 each) prior to the ultimate experimental analysis. A separate group of rats was spared the operation to be used as controls (n=4) (indicated by an empty circle). All animals (operated as well as controls) were subjected to the ultimate experimental analysis (inicated by double, capped vertical lines), comprising a pentobarbital sleeping test, followed by the sacrificing of the animal and the harvest of liver lobes for later laboratory analysis. (b)

Figure 2. Pentobarbital sleeping test. A sleeping test with an intraperitoneal injection of pentobarbital (30 mg/body weight kilogram) was performed on control rats, as well as on animals subjected to portal vein ligation (PVL) and different survival periods. Statistical analysis was performed with analysis of variance (ANOVA) with Bonferroni's *post hoc* test to correct for multiple comparisons. Results are given as means ± standard deviation, representing n=4 animals per time points. '*' indicates $P< 0.05$ for actual versus control values.

Figure 3. Liver lobe weights. The individual weights of ligated and non-ligated liver lobes were assessed following portal vein ligation (PVL) and in control animals, and ultimately expressed both as per cent of total liver weight, (a) and per cent of body weight. (b) Statistical analysis was performed with analysis of variance (ANOVA) with Bonferroni's *post hoc* test to correct for multiple comparisons. Results are given as means ± standard deviation, representing n=4 animals per time points. '*', '&' and 'σ' indicate $p < 0.05$ for actual versus control values of ligated lobes, non-ligated lobes or total liver, respectively. '#' indicates $P < 0.05$ ligated lobes versus contemporary non-ligated lobes.

Figure 4. CYP1A enzyme activity and CYP1A2 mRNA expression. Cytochrome P450 (CYP) 1A enzyme activity, (a, b) as well as corresponding CYP1A2 mRNA expression (c) of ligated and non-ligated liver lobes were assessed following portal vein ligation (PVL) and in control animals. CYP1A enzyme activity was represented by the characteristic O-deethylation of ethoxyresorufin, and ultimately expressed as both 'intrinsic' activity per mg of microsomal protein, (a) as well as the total activity of ligated and non-ligated lobes. (b) Statistical analysis was performed with analysis of variance (ANOVA) with Bonferroni's *post hoc* test to correct for multiple comparisons. Results are given as means ± standard deviation, representing n=4 animals per time points. '*', '&' and 'σ' indicate $p < 0.05$ for actual versus control values of ligated lobes, non-ligated lobes or total liver, respectively. '#' indicates $P < 0.05$ ligated lobes versus contemporary non-ligated lobes.

Figure 5. CYP2B enzyme activity and CYP2B1/2 mRNA expression. Cytochrome P450 (CYP) 2B enzyme activity, (a, b) as well as corresponding CYP2B1 and CYP2B2 mRNA expression (c, d) of ligated and non-ligated liver lobes were assessed following portal vein ligation (PVL) and in control animals. CYP2B enzyme activity was represented by the characteristic O-dealkylation of pentoxyresorufin, and ultimately expressed as both 'intrinsic' activity per mg of microsomal protein, (a) as well as the total extrapolated activity of ligated

and non-ligated lobes. (b) Statistical analysis was performed with analysis of variance (ANOVA) with Bonferroni's *post hoc* test to correct for multiple comparisons. Results are given as means ± standard deviation, representing n=4 animals per time points. '*', '&' and 'σ' indicate p < at least 0.05 for actual versus control values of ligated lobes, non-ligated lobes or total liver, respectively. '#' indicates *P*< 0.05 ligated lobes versus contemporary non-ligated lobes.

Figure 6. CYP2C enzyme activity and CYP2C6, CYP2C11, CYP2C13 mRNA expression. Cytochrome P450 (CYP) 2C enzyme activity, (a, b) as well as corresponding CYP2C6, CYP2C11 and CYP2C13 mRNA expression (c-e) of ligated and non-ligated liver lobes were assessed following portal vein ligation (PVL) and in control animals. CYP2C enzyme activity was represented by the characteristic 4-hydroxylation of tolbutamide, and ultimately expressed as both 'intrinsic' activity per mg of microsomal protein, (a) as well as the total extrapolated activity of ligated and non-ligated lobes. (b) Statistical analysis was performed with analysis of variance (ANOVA) with Bonferroni's *post hoc* test to correct for multiple comparisons. Results are given as means ± standard deviation, representing n=4 animals per time points. '*', '&' and 'σ' indicate *P*< 0.05 for actual versus control values of ligated lobes, non-ligated lobes or total liver, respectively. '#' indicates *P*< 0.05 ligated lobes versus contemporary non-ligated lobes.

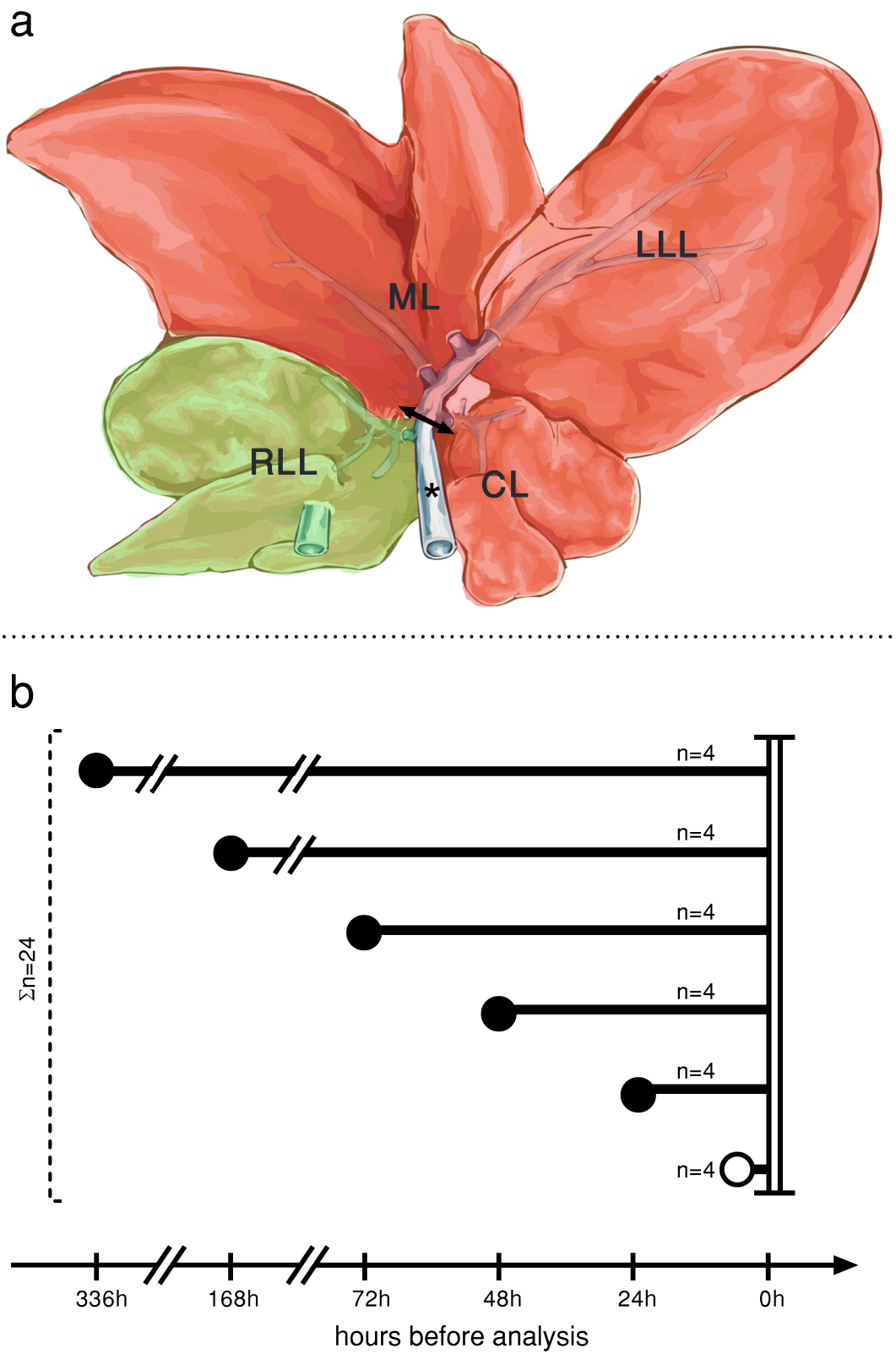
Figure 7. CYP3A enzyme activity and CYP3A1 mRNA expression. CYP3A enzyme activity, (a-d) as well as corresponding CYP3A1 mRNA expression (e) of ligated and non-ligated liver lobes were assessed following portal vein ligation (PVL) and in control animals. CYP3A enzyme activity was represented by the characteristic 1'- and 4-hydroxylation of midazolam, and ultimately expressed as both 'intrinsic' activity per mg of microsomal protein, (a, c) as well as the total activity of ligated and non-ligated lobes. (b, d) Statistical analysis was performed with analysis of variance (ANOVA) with Bonferroni's *post hoc* test

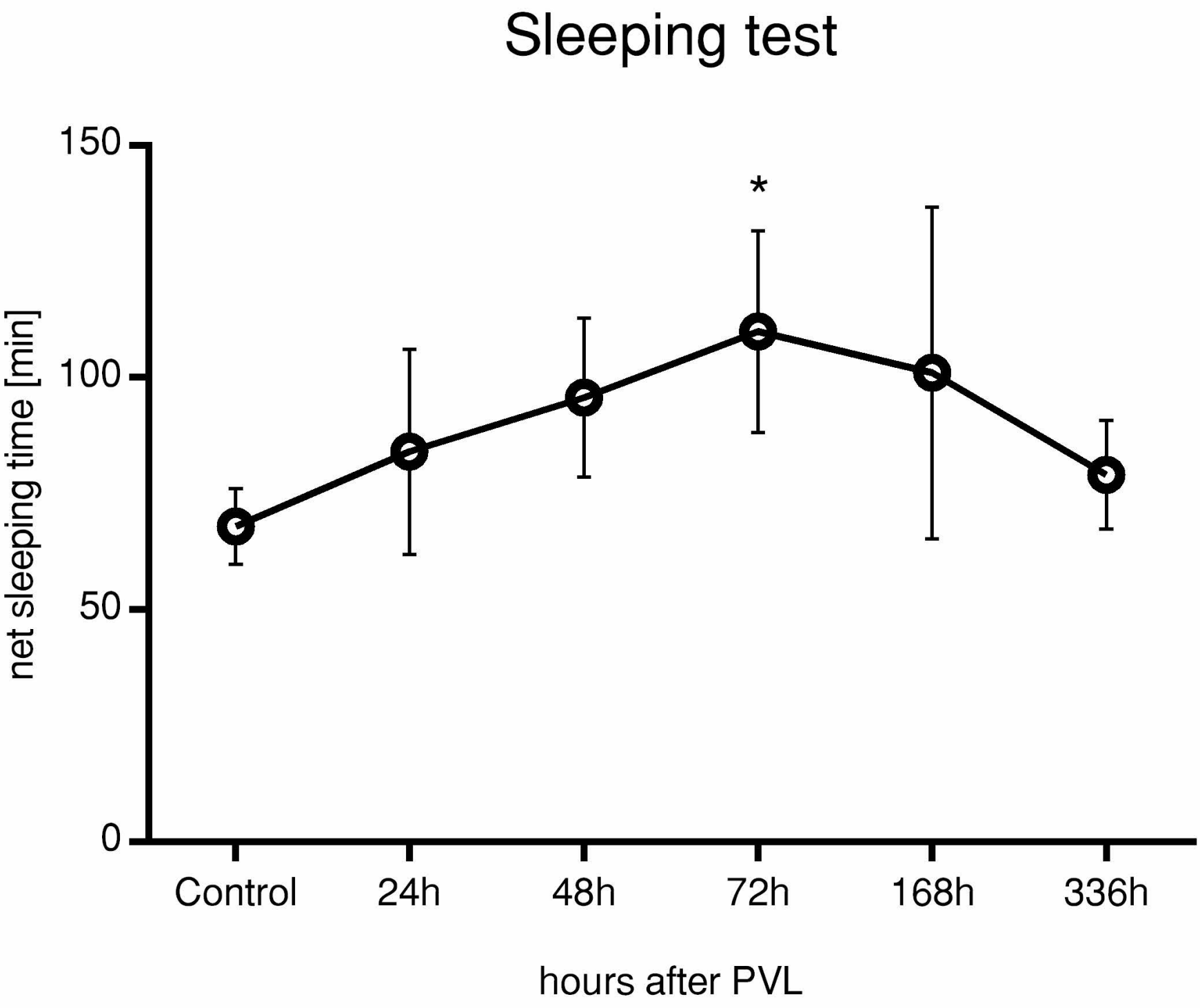
to correct for multiple comparisons. Results are given as means ± standard deviation, representing n=4 animals per time points. '*', '&' and 'σ' indicate $p < 0.05$ for actual versus control values of ligated lobes, non-ligated lobes or total liver, respectively. '#' indicates $P < 0.05$ ligated lobes versus contemporary non-ligated lobes.

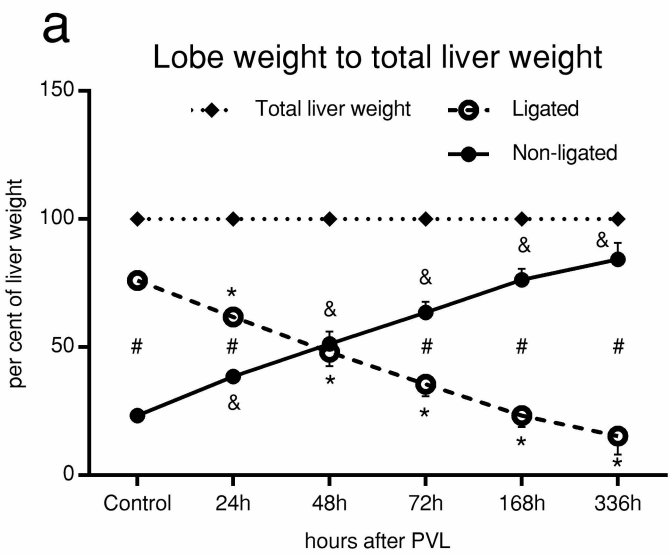
Figure 8. CYP3A1 immunohistochemical reaction. Standard immunohistochemical reactions were performed on formaldehyde-fixed-paraffin-embedded sections of ligated and non-ligated liver specimens of animals subjected to portal vein ligation (PVL) (b-f, h-l) and controls (a, g). The reactions were simultaneously processed using identical protocols in an automated slide stainer, with primary antibodies against Cytochrome P450 (CYP) 3A1 and standard labelling based on horseradish-peroxidase-diaminobenzidine interaction. On all sections, CYP3A1 protein expression displayed a uniform pericentral localization. White bars represent 200 µm.

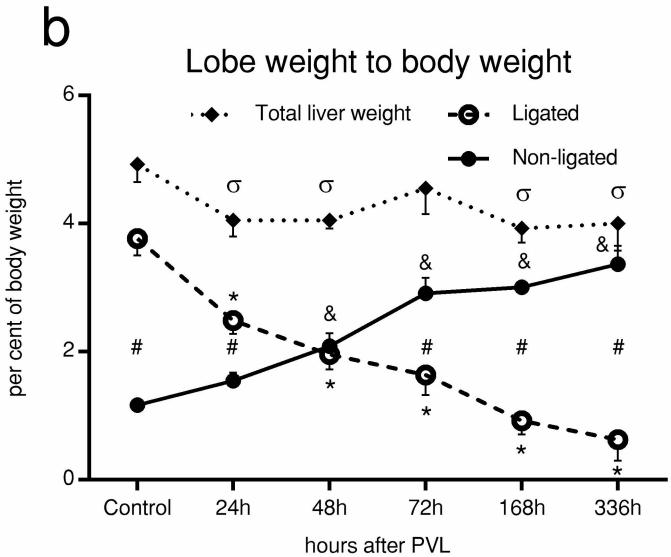
Table 1. Primer sequences. Sequences of polymerase chain reaction (PCR) primers for cytochrome P450 (CYP) enzymes and hypoxanthine phosphoribosyltransferase (HPRT).

Gene	5'→3'Sequence	Primers	Amplicon size (bp)
CYP1A2	Forward	CCTACAACTCTGCCAGTCTCC	89
	Reverse	AGTAGCAGCTCTGGGGCTAA	
CYP2B1	Forward	CTCCTCGTGGGCTTCTTGTT	149
	Reverse	TCTCGAAGCTGCATGAAGGAA	
CYP2B2	Forward	TGCATGGATGAGAGAGGAGAAG	134
	Reverse	TGAGCAGGAAACCATAGCGG	
CYP2C6	Forward	AGCAGTCCCTGGACACAGTT	205
	Reverse	TGTGATTTTCCTGCTTCCACTT	
CYP2C11	Forward	TGTTTGGAGCTGGCACAGAA	88
	Reverse	ACTTTAGCTGTGACATCCACG	
CYP2C13	Forward	TTCTCAGCAGGAAAACGGATGT	169
	Reverse	GGAAAGTGGGAGGAACTGAAGAA	
CYP3A1	Forward	ACCAGCAGCACACTTTCCTT	106
	Reverse	GAGGTGCCTTATTGGGCAGA	
HPRT	Forward	CTCATGGACTGATTATGGACAGGAC	123
	Reverse	GCAGGTCAGCAAAGAACTTATAGCC	

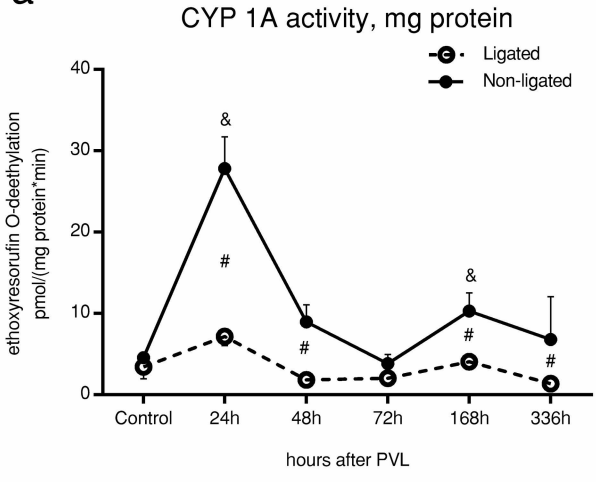

a
LLL
ML
RLL
CL
*
b
n=4
n=4
n=4
n=4
n=4
n=4
Σn=24
336h
168h
72h
48h
24h
0h
hours before analysis


Sleeping test
150
100
50
0
net sleeping time [min]
*
Control
24h
48h
72h
168h
336h
hours after PVL

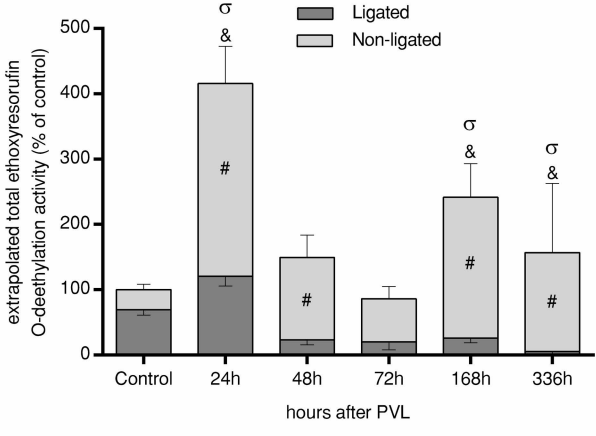

a
Lobe weight to total liver weight
Total liver weight
Ligated
Non-ligated
per cent of liver weight
150
100
50
0
Control
24h
48h
72h
168h
336h
hours after PVL


b
Lobe weight to body weight
Total liver weight
Ligated
Non-ligated
per cent of body weight
6
4
2
0
Control
24h
48h
72h
168h
336h
hours after PVL

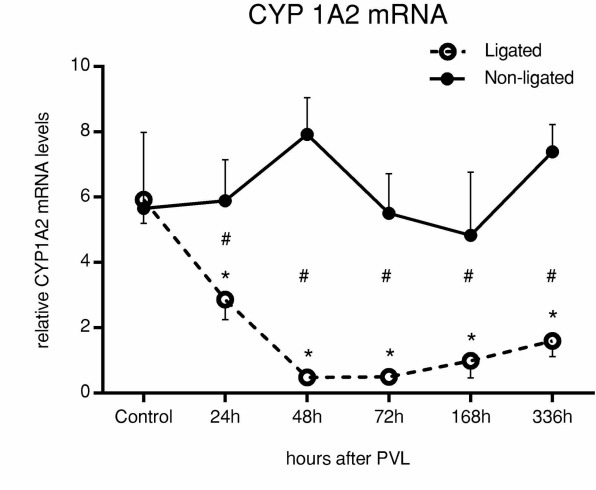
a

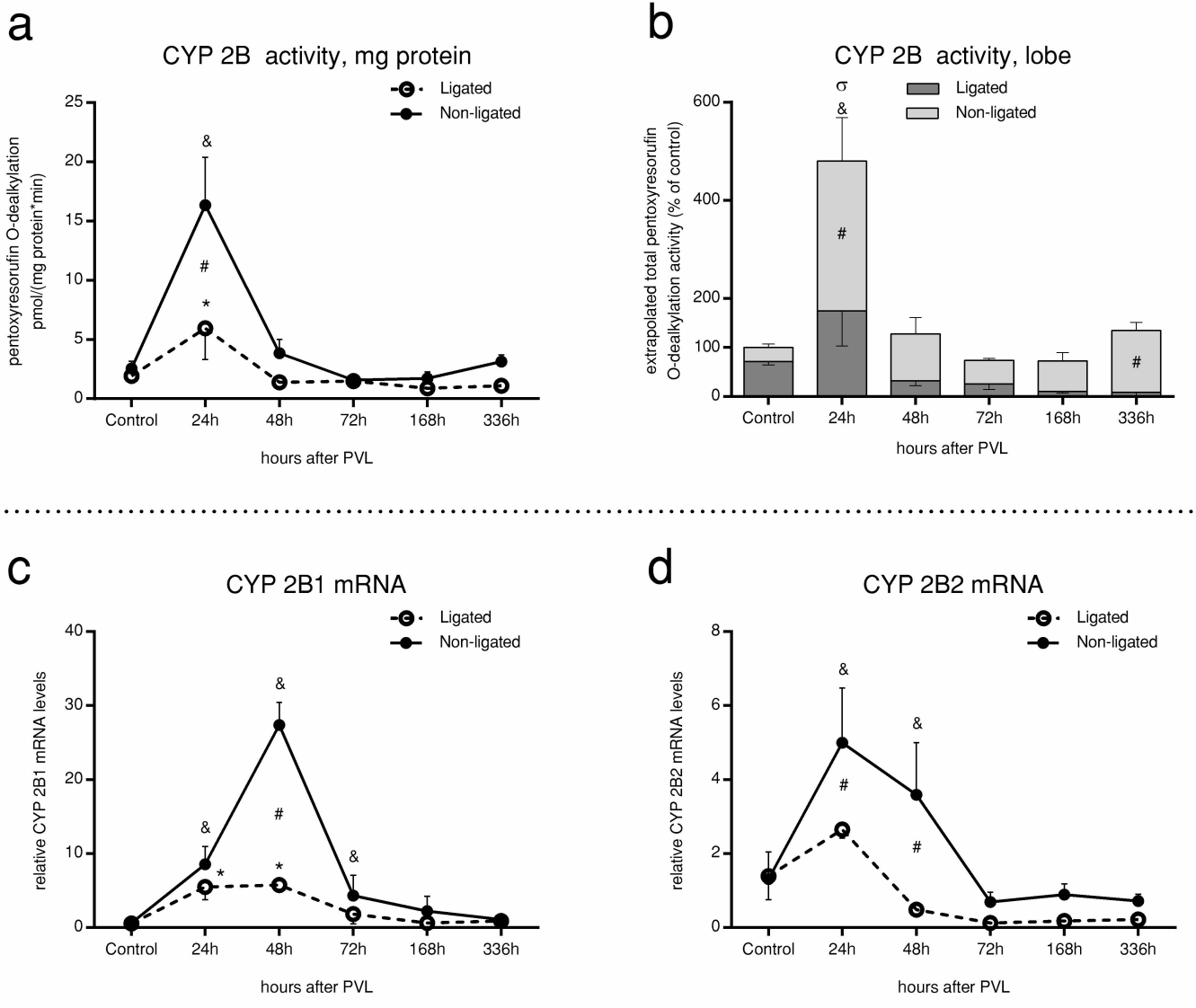

CYP 1A activity, mg protein
Ligated
Non-ligated
ethoxyresorufin O-deethylation
pmol/(mg protein*min)
40
30
20
10
0
&
#
Control
24h
48h
72h
168h
336h
hours after PVL

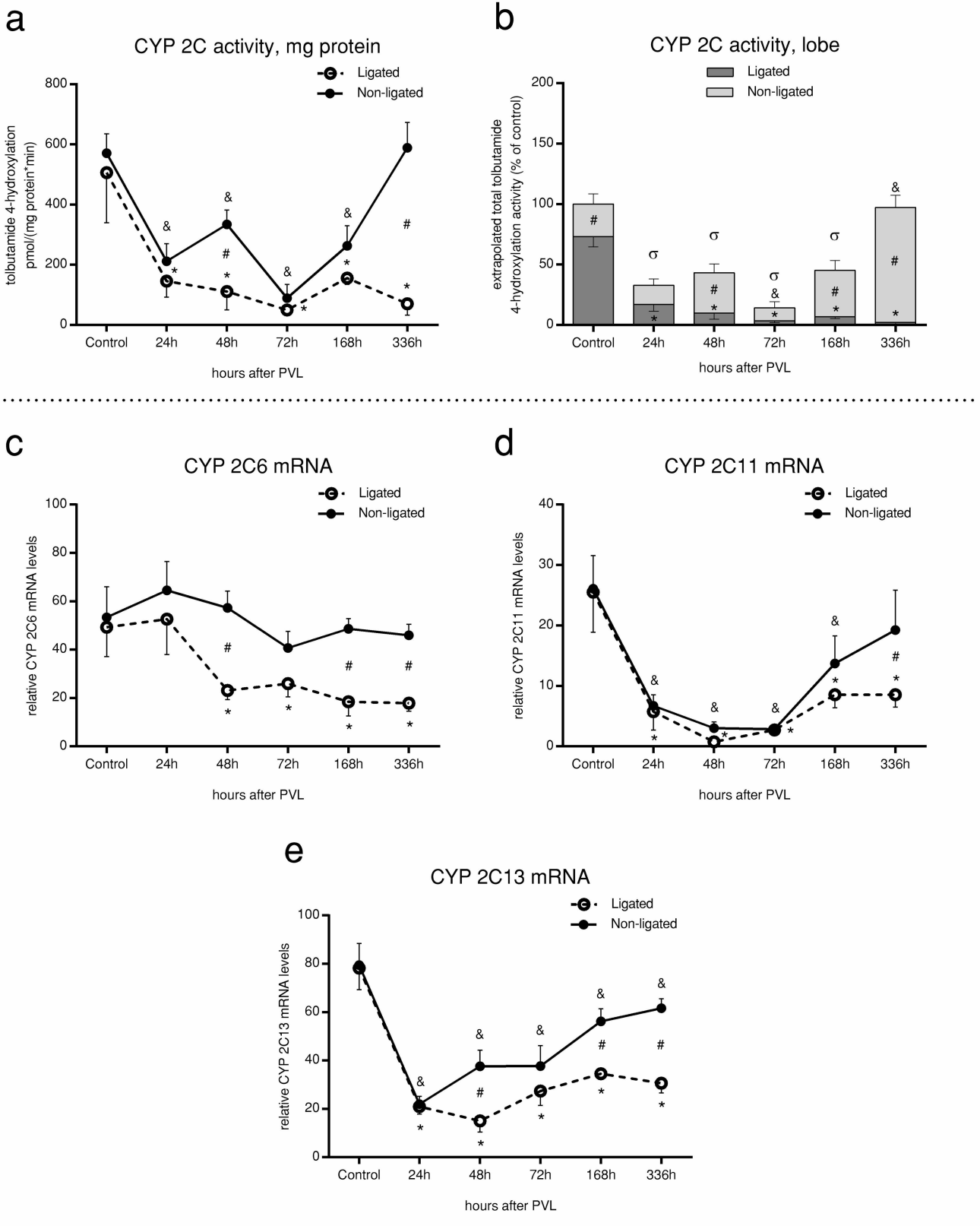
b

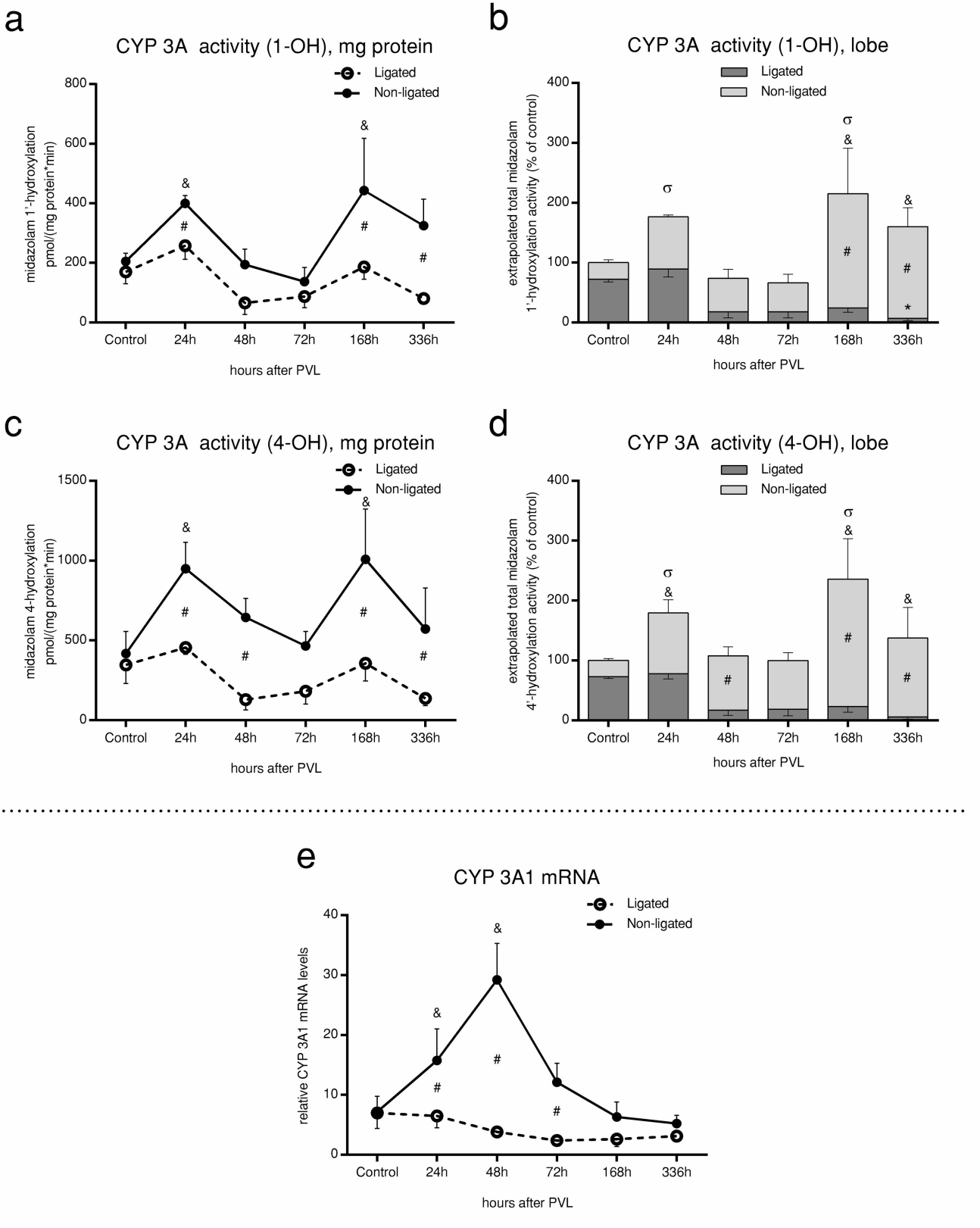

CYP 1A activity, lobe
Ligated
Non-ligated
extrapolated total ethoxyresorufin
O-deethylation activity (% of control)
500
400
300
200
100
0
σ
&
#
Control
24h
48h
72h
168h
336h
hours after PVL

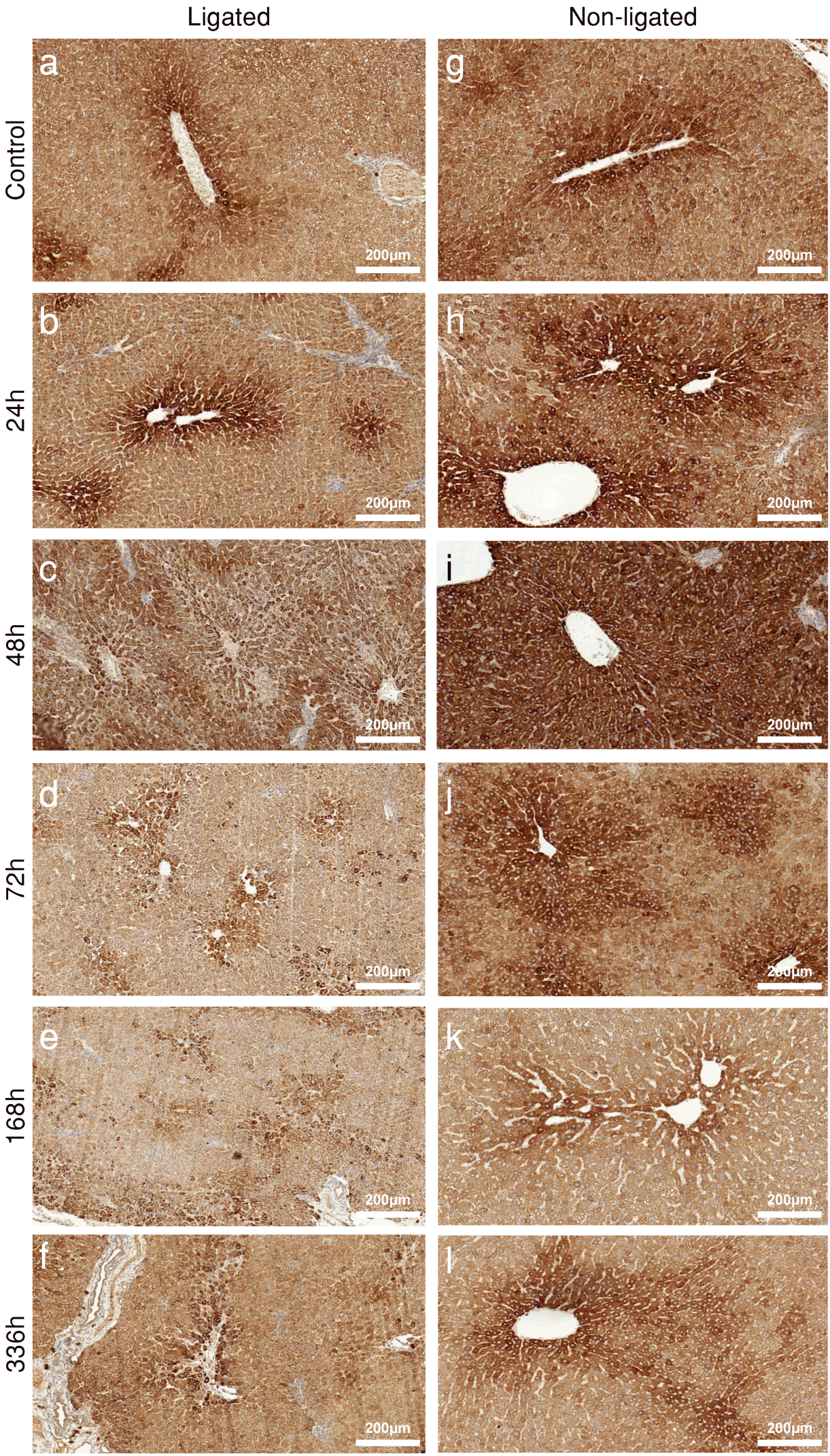
c


CYP 1A2 mRNA
Ligated
Non-ligated
relative CYP1A2 mRNA levels
10
8
6
4
2
0
#
*
Control
24h
48h
72h
168h
336h
hours after PVL


a
CYP 2B activity, mg protein
Ligated
Non-ligated
pentoxyresorufin O-dealkylation
pmol/(mg protein*min)
25
20
15
10
5
0
&
#
*
Control
24h
48h
72h
168h
336h
hours after PVL
b
CYP 2B activity, lobe
Ligated
Non-ligated
extrapolated total pentoxyresorufin
O-dealkylation activity (% of control)
600
400
200
100
0
σ
&
#
#
Control
24h
48h
72h
168h
336h
hours after PVL
c
CYP 2B1 mRNA
Ligated
Non-ligated
relative CYP 2B1 mRNA levels
40
30
20
10
0
&
&
&
#
*
*
Control
24h
48h
72h
168h
336h
hours after PVL
d
CYP 2B2 mRNA
Ligated
Non-ligated
relative CYP 2B2 mRNA levels
8
6
4
2
0
&
&
#
#
Control
24h
48h
72h
168h
336h
hours after PVL


a
CYP 2C activity, mg protein
Ligated
Non-ligated
tolbutamide 4-hydroxylation
pmol/(mg protein*min)
800
600
400
200
0
Control
24h
48h
72h
168h
336h
hours after PVL
b
CYP 2C activity, lobe
Ligated
Non-ligated
extrapolated total tolbutamide
4-hydroxylation activity (% of control)
200
150
100
50
0
Control
24h
48h
72h
168h
336h
hours after PVL
c
CYP 2C6 mRNA
Ligated
Non-ligated
relative CYP 2C6 mRNA levels
100
80
60
40
20
0
Control
24h
48h
72h
168h
336h
hours after PVL
d
CYP 2C11 mRNA
Ligated
Non-ligated
relative CYP 2C11 mRNA levels
40
30
20
10
0
Control
24h
48h
72h
168h
336h
hours after PVL
e
CYP 2C13 mRNA
Ligated
Non-ligated
relative CYP 2C13 mRNA levels
100
80
60
40
20
0
Control
24h
48h
72h
168h
336h
hours after PVL


a
CYP 3A activity (1-OH), mg protein
Ligated
Non-ligated
midazolam 1'-hydroxylation
pmol/(mg protein*min)
800
600
400
200
0
Control
24h
48h
72h
168h
336h
hours after PVL
b
CYP 3A activity (1-OH), lobe
Ligated
Non-ligated
extrapolated total midazolam
1'-hydroxylation activity (% of control)
400
300
200
100
0
Control
24h
48h
72h
168h
336h
hours after PVL
c
CYP 3A activity (4-OH), mg protein
Ligated
Non-ligated
midazolam 4-hydroxylation
pmol/(mg protein*min)
1500
1000
500
0
Control
24h
48h
72h
168h
336h
hours after PVL
d
CYP 3A activity (4-OH), lobe
Ligated
Non-ligated
extrapolated total midazolam
4'-hydroxylation activity (% of control)
400
300
200
100
0
Control
24h
48h
72h
168h
336h
hours after PVL
e
CYP 3A1 mRNA
Ligated
Non-ligated
relative CYP 3A1 mRNA levels
40
30
20
10
0
Control
24h
48h
72h
168h
336h
hours after PVL


Ligated
Non-ligated
Control
24h
48h
72h
168h
336h
a
b
c
d
e
f
g
h
i
j
k
l
200μm