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Katabolická dráha hemu v patogenezi jaterních onemocnění

Heme catabolic pathway in pathogenesis of liver diseases

Disertační práce/Doctoral thesis

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Abstrakt

Předkládaná práce se zabývá rolí katabolické dráhy hemu v patogenezi vybraných jaterních onemocnění. Cílem bylo objasnit, zda může modulace enzymu hemoxygenázy (Hmox) a vznikajících produktů- zvláště oxidu uhelnatého (CO) a bilirubinu- ovlivnit vznik a průběh zánětlivých a cholestatických pochodů v játrech.

U modelu zánětu vyvolaného podáním endotoxinu se nám podařilo prokázat, že indukce *hmox1* chrání jaterní tkáň před hepatocelulárním poškozením. Podávání CO potkanům s cholestázou indukovanou endotoxinem mělo za následek snížení exprese zánětlivých cytokinů v jaterní tkáni a zároveň zabránilo snížení exprese jaterních transportérů, což vedlo k významným hepatoprotektivním účinkům. Dále jsme jako první popsali *in vivo* distribuci a eliminaci inhalovaného CO ve tkáních potkana.

V *in vitro* experimentech a u modelu extrahepatální cholestázy jsme sledovali roli bilirubinu v ochraně hepatocytů před oxidačním poškozením. Rovněž jsme prokázali, že indukce *hmox1* zvyšuje expresi jaterních transportérů u modelu cholestázy indukované estrogeny, což vede ke stimulaci odtoku žluče a normalizaci plazmatických markerů cholestázy a jaterního poškození.

Výsledky předložené práce dokazují, že katabolická dráha hemu se významně zapojuje do patogeneze cholestatických a zánětlivých onemocnění, a regulace této dráhy by mohla vést k vývoji nových terapeutických postupů.

Klíčová slova: hemoxygenáza, hem, oxid uhelnatý, bilirubin, jaterní nemoci, cholestáza

Abstract

This thesis focuses on the role of heme catabolic pathway in the pathogenesis of selected liver diseases. The aim was to clarify if the modulation of heme oxygenase (Hmox) and its catabolic products – especially carbon monoxide (CO) and bilirubin – affected the development and progression of liver diseases, focusing on the inflammatory and cholestatic pathways.

Firstly, we discovered that the induction of *hmox1* prevented hepatocellular damage in endotoxin-induced inflammation. Furthermore, administration of CO *in vivo* in early-phase of endotoxin-induced cholestasis decreased the inflammatory cytokine production in the liver and simultaneously prevented downregulatory effect of cytokines on hepatocyte transporters resulting in hepatoprotection. For the first time, we characterized *in vivo* tissue distribution and elimination of inhaled CO in rats.

In vitro experiments and the model of extrahepatic cholestasis revealed the significant role of intracellular bilirubin in hepatocellular protection against oxidative damage, which accompanies cholestatic disorders. Last but not least, *hmox1* induction by heme increased hepatocyte transporters expression and subsequently stimulated bile flow participating in conferring protection against estrogen-induced cholestasis.

Presented results demonstrate that the heme catabolic pathway is significantly involved in the cholestatic and inflammatory pathways in the liver, and its modulation might represent a potential therapeutical strategy for the treatment of liver diseases.

Key words: heme oxygenase, heme, carbon monoxide, bilirubin, liver diseases, cholestasis

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Content

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1 INTRODUCTION

1.1 Heme Catabolic Pathway

1.1.1 Heme and its catabolic pathway

The term heme refers to a complex of a metal ion (Fe, Mg, Mn, Zn, Sn, Cd, Co, Cu, Cr, and Ag) chelated in a porphyrin ring. The porfyrin ring is protoporphyrin IX. The term, however, is customarily used in reference to the iron complex of such chelates. The capacity of the chelated metal ion to undergo a reversible change in the oxidation state (e.g. $Fe^{2+} \rightarrow Fe^{3+}$) renders heme compounds effective biological catalysts [1].

Most mammalian cells contain a „free“ heme pool, i.e. nonprotein bound heme, providing heme for the synthesis of heme-containing proteins and possibly for carbon monoxide (CO) production [2]. However, when free heme exceeds the physiological range, its cytotoxic role dominates its constitutive role in heme protein formation. An abnormally high level of heme in organism is connected to many diseases caused by oxidative stress and thus this molecule needs to be properly metabolized [3].

The free heme is carried by hemopexin or albumin to the reticuloendothelial system. In the reticuloendothelial system, heme oxygenase (HMOX) acts as the rate-limiting enzyme in heme degradation [4]. HMOX forms a complex with NADPH-dependent flavoprotein reductase (cytochrome *P450* reductase) and biliverdin reductase (a cytosolic enzyme, BLVRA) on the endoplasmic reticulum (ER). In the presence of functional HMOX, the porphyrin ring of heme (ferroprotoporphyrin IX) is broken and oxidized at the α-methen bridge, producing equimolar amounts of CO, ferrous ion, and biliverdin that is immediately metabolized to bilirubin by BLVRA. The heme catabolic pathway, as depicted in Fig.1, also requires a molecule of NADPH and oxygen. HMOX

activity in different types of cells is also largely responsible for the degradation of heme derived from denaturated heme proteins [5].

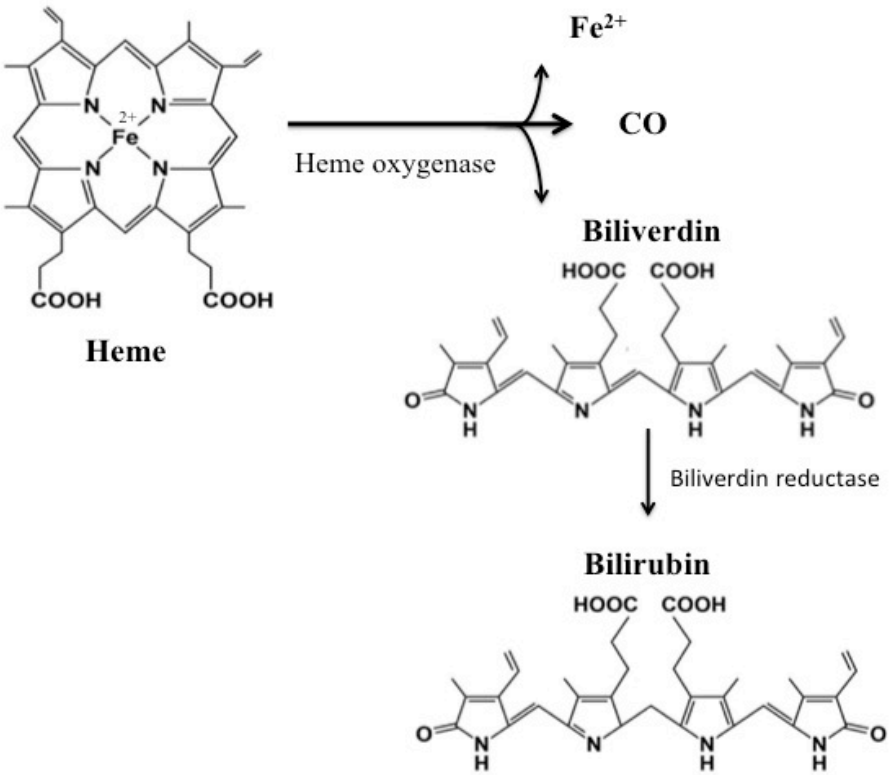

Fig.1. Heme catabolic pathway. *Heme oxygenase, the rate-limiting enzyme in the heme catabolic pathway, utilizes cytochrome P450, NADPH and molecular oxygen to cleave the heme ring on α-methen bridge, forming biliverdin, CO and ferrous iron. Biliverdin is subsequently converted to bilirubin by biliverdin reductase.*

1.1.2 Heme oxygenase

HMOX (E.C 1.14.99.3) is the rate-limiting enzyme in the heme catabolic pathway. Two genetically distinct isoformes of HMOX have been described to this date: an inducible form, heme oxygenase-1 (HMOX1), and a constitutively expressed form, heme oxygenase-2 (HMOX2). The putative third isoform, heme oxygenase-3 (HMOX3), does not have an evident effect in organism as it seems to be a pseudogene of *HMOX2* [6].

The inducible isoform, Hmox1, was discovered in 1974 in two independent laboratories [7, 8] and is traditionally illustrated as a microsomal protein with a primary localization in ER. Subcellular localization of Hmox1 has also been detected in the cytoplasm, nuclear matrix, mitochondria, and peroxisomes of parenchymal and nonparenchymal liver cell population [6]. The highest expression of *Hmox1* can be seen in the spleen. In fact, under physiological conditions, the spleen is the only organ in which *Hmox1* overpowers *Hmox2* [9].

However, the *Hmox1* gene is one of the most inducible genes that has been described, and it can be stimulated by many factors connected to intracellular redox system imbalance, including heme, heavy metals [10], UV radiation, sodium arsenite, reactive oxygen species (ROS) [11], ethanol [12], curcumin [13], metalloporphyrins [14], nitric oxide [15], and lipopolysaccharide (LPS) [16]. To less content, negative regulators have been described, e.g. N-acetylcysteine reducing *Hmox1* induction by ROS [17], angiotensin II via calcium-signaling [18], interferon γ (IFNγ) [19], and prostaglandin E2 inhibiting overexpression of *Hmox1* induced by interleukin-1β [20]. Thanks to impressive number of molecules functioning via the Hmox1 system, Hmox1 has been previously described as a "therapeutic funnel" [54].

The control of *Hmox1* expression is a complex process tightly regulated by a number of response elements (REs), sequence-specific DNA binding proteins (Bach1, transcription factors) and signal transduction pathways. Stimulation of the *Hmox1* gene is primarily controlled at the transcriptional level by REs localized in the promoter 5′-flanking region of *Hmox1* including activator protein-1 (AP-1), metal responsive element, oncogene c-myc/max heterodimer binding site (Myc/Max), and antioxidant response element [21]. Many transcriptional factors involved in *Hmox1* transcription have been identified as stress response elements (StREs)-binding proteins including

individual members of Jun, Fos, cAMP response element binding protein, activating transcription factor, Maf and Cap'N'Collar-basic-leucin zipper families. The very last family includes *Hmox1* expression activating nuclear factor-erythroid related factor 2 (Nrf2) and its antagonist Bach1 [22]. Under basal conditions, Bach1 forms heterodimers with small proteins of the Maf family, which can bind to *Hmox1* promotor resulting in repression of transcription. Depleting Bach1 by heme allows activating heterodimers to increase transcription of *Hmox1* [23] (Fig.2). Among signaling transduction pathways, mitogen-activated protein kinases (MAPKs) including c-Jun N-terminal kinase (JNK) [24], extracellular signal-regulated protein kinase (ERK), and p38 mitogen activated protein kinase (p38 MAPK) [25]; protein kinase C [26], protein phosphatases [27], and phosphatidylinositol 3-kinase (PI3K) [28] have been found to mediate *Hmox1* upregulation.

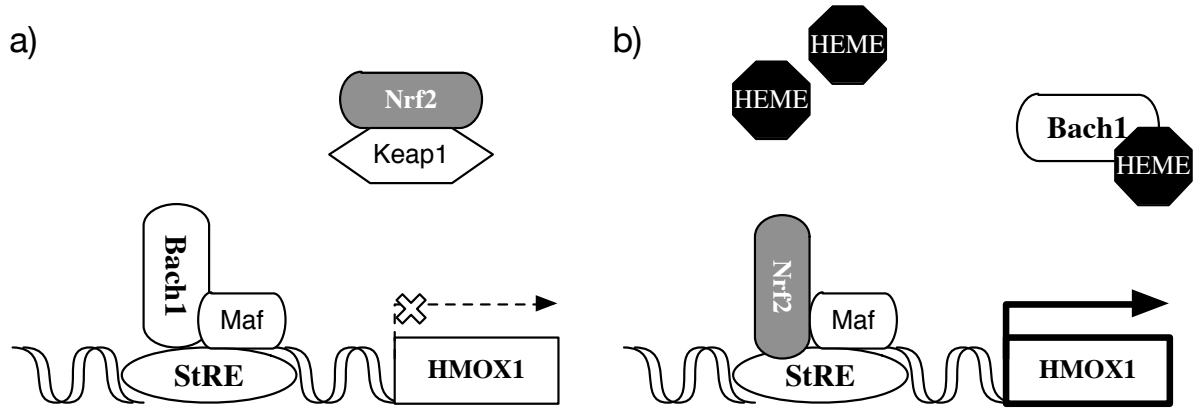

Fig.2. Regulation of HMOX1 expression via Nrf2/Bach1. *a) Under basal conditions, Bach1 binds to StRE of the promoter region of HMOX1 gene that prevents Nrf2 from binding to promoter and transcription of HMOX1. b) Heme depletes Bach1 from its binding site making StRE available for Nrf2, which leads to transcription of HMOX1.*

In 1986, Maine´s laboratory identified the constitutive Hmox2 isoform from rat liver microsomes [29]. *Hmox2* is predominantly expressed in the brain and testes and also constitutively expressed in other tissues, including endothelium, distal nephron segments, myenteric plexus of the gut, and in other tissues at low levels [9]. Hmox2 proteins are anchored to ER by a hydrophobic sequence of amino acids at the carboxyl terminus of the protein [30].

The physiological importance of Hmox1 has been confirmed by the phenotypic characterization of Hmox1-deficient mice [31] and the first documented case of a patient with HMOX1 deficiency [32]. The HMOX1 deficient patient suffered from severe growth retardation and persistent hemolytic anemia characterized by a marked intravascular hemolysis, which was associated with low levels of bilirubin, an abnormal coagulation/fibrinolysis system, and persistent endothelial damage. Lymphoblastoid cells revealed a complete absence of HMOX1 production and were extremely sensitive to oxidant-induced cell injury [32]. Growth retardation, anemia, iron deposition, and vulnerability to stress injuries are all characteristics observed in previously described *hmox1* knockout mice [31]. In contrast to the severe phenotypes of *hmox1*$^{-/-}$ mice, *hmox2* deficient mice are fertile and survive normally for at least one year [33]. Subsequent studies have revealed the increased susceptibility to hyperoxic lung damage and iron accumulation in *hmox2*$^{-/-}$ mice even though the expression of *hmox1* was increased by two folds [34]. Thus, Hmox1 does not necessary compensate for Hmox2 function.

1.1.3 Products of heme catabolic pathway

1.1.3.1 Carbon monoxide

CO is the diatomic oxide of carbon. CO is a colorless, odorless and tasteless gas that is, to a considerable extent, chemically stable. The coordinated CO has greater reactivity than the free gas, and the reduction of CO can be greatly facilitated by transition metals. Once formed, metal carbonyls are relatively stable until CO is displaced, e.g. by molecular oxygen [5].

On the contrary to the hazardous impact of its intoxication, CO is an ubiquitous and essential endogenous signaling molecule in mammalian system. CO is constantly produced in the heme degradation pathway by constitutive HMOX2 and inducible HMOX1 in ER of many cell types. Its ability to diffuse through membranes makes CO a fast signaling molecule. To this date, several signal transduction pathways including soluble guanylate cyclase (sGC) activation [35], modulation of inducible nitric oxide synthase (iNOS) [15, 36], regulation of MAPKs [37, 38], PI3K/Akt pathway [39, 40], signal transducers and activators of transcription (STATs) [39], peroxisome proliferator-activated receptor-γ [41], cystathionine beta-synthase [42], and stabilization of hypoxia-inducible factor 1-α [43] were recognized.

Despite the earlier concept that CO is only waste for mammalian organisms that are oxygen transport dependent, the physiological role of endogenously produced CO seems to be important. Since the extensive studies on the vasoactive effects of CO due to similarity to NO mediated via sGC pathway [44, 45], many biological actions have been uncovered. CO with its ability to inhibit pro-inflammatory genes and augment anti-inflammatory cytokine production by the activation of several MAPK [37, 46, 47] and sGC pathway [47] acts as an anti-inflammatory agent. Furthermore, CO can protect cells against apoptosis via MAPK signaling [39, 48, 49] and provides cytoprotection by

inducing nuclear factor-kappaB (NF-κB)-mediated NO production in TNF-induced hepatotocyte cell death [15]. Recently, we have shown that CO mediates antiproliferative effects by inhibiting Akt phosphorylation in pancreatic cancer cells [40]. The CO-mediated activation of sGC and subsequent cGMP production may also contribute to reduction in cancer proliferation [50] (Fig.3).

CO has been shown to inhibit platelet aggregation by increasing cGMP [51]. Interestingly, CO delivered by CO-releasing molecules inhibits platelet aggregation in the absence of sGC activation [52]. Taken together with antioxidant and vasoactive effects, CO displays anti-atherogenic properties [53] (Fig.3). This variety of CO actions coordinates with the key protective role of *hmox1* induction in different stress conditions [54].

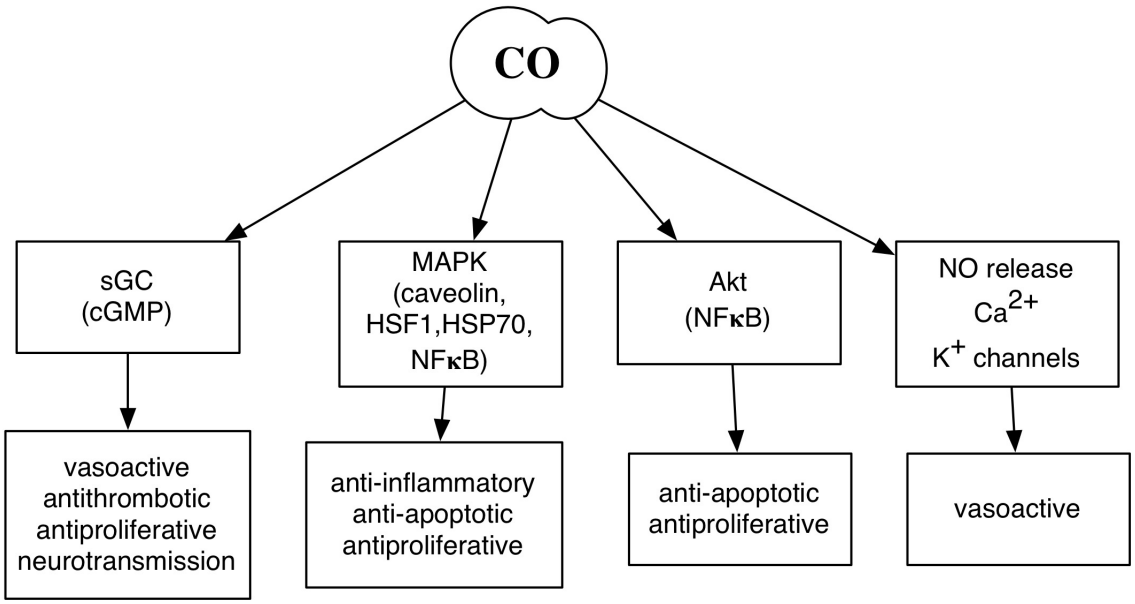

Fig.3. Carbon monoxide signal transduction pathways*. CO displays various physiological effects via different transduction pathways including sGC, MAPK, Akt and NO- and Ca2+-dependent pathways.*

Although the evidence on the positive actions of CO in organisms is convincing, the administration of gaseous molecules is strongly limited by the formation of carbonylhemoglobin. Recently, metal-based carbon monoxide releasing compounds (CORMs) have been developed providing a pharmacological method for delivery of CO as a promising alternative to inhalation [55].

1.1.3.2 Biliverdin/Bilirubin

HMOX catalyzes the degradation of a potential pro-oxidative molecule of heme to form a molecule of biliverdin (BVR). BVR is immediately transformed by another cytoprotective enzyme BLVRA (E.C 1.3.1.24) to bilirubin. Bilirubin, the principal bile pigment, is the end product of heme catabolism. For many years, bilirubin was thought to have no physiological function other than as a waste product of heme catabolism. Although severe hyperbilirubinemia in neonates has been shown to be neurotoxic, studies performed during the past decade have found that bilirubin has a number of interesting biochemical and biological properties [56].

BLVRA, which converts BVR to bilirubin, contributes to cell signaling by its translocation from cytosol into the nucleus where it functions in a variety of transcriptional factors and signaling pathways regulation [57]. These pathways include AP-1-regulated genes, transforming growth factor-β (TGF-β), NF-κB, janus kinase/signal transducer and activator of transcription (JAK/STAT) and MAPK signaling pathways [58-60]. Anti-inflammatory effects of BVR are mediated via PI3K/Akt pathway [61].

In the liver, bilirubin is conjugated by bilirubin UDP-glucuronosyl transferase (UGT1A1, E.C 2.4.1.17) and secreted via the active transporter multidrug resistance-associated protein-2 (MRP2, *ABCC2,* OMIM*601107) into bile. Certain polymorphism

in *UGT1A1* leads to the manifestation of unconjugated hyperbilirubinemia called Gilbert syndrome [62]. Interestingly, a number of clinical studies have shown that subjects with this condition ale less likely to develop atherosclerosis [63], colorectal cancer [64, 65] and chronic inflammation [56].

In 1987, Stocker and his colleagues have shown that albumin-bound bilirubin at concentrations found in human plasma is a very efficient peroxyl radical scavenger and can protect albumin-transported fatty acids from the oxidation by these radicals [66]. In fact, both unconjugated and conjugated bilirubin can protect human low density lipoprotein (LDL) against oxidation [67]. Altogether, bilirubin and BVR may play an important role in the development of atherosclerosis [53, 68]. Subsequently, many studies have confirmed the antioxidant potential of bilirubin [69-71]. Low nanomolar concentration of unconjugated bilirubin can protect cells against 10,000-fold higher concentration of hydrogen peroxide. The study reported that this unique protective effect is mediated by BVR-bilirubin redox cycle, in which bilirubin is oxidized to BVR and then recycled by BLVRA back to bilirubin. This amplification allows bilirubin to be ranked among the most potent antioxidant in nature [71]. However, the results of recent studies suggest other mechanisms of BVR formation [72] and argue against the BVR-mediated redox cycle as an important mechanism in cellular antioxidant defense [73].

To this date, several groups of bilirubin oxidative products (BOXes) have been described. BOXes have been reported to be involved in the diseases, however the experimental prove is hard to obtain due to its high light sensitivity. Research on the chemical characteristics of these compounds and their relevancy in human disease pathogenesis is presently under intensive investigation [74].

BVR and bilirubin were also shown to protect tissues against inflammatory damage [75-77]. Treatment with BVR injections has been shown to selectively modulate the inflammatory cascade and decrease neutrophil infiltration in polymicrobial sepsis [77]. Furthermore, bilirubin partly inhibits iNOS expression and suppresses NO production that is considered one of the key mediators in endotoxemia [76]. Identically, BVR has been reported to inhibit NO, pro-inflammatory cytokines and cell adhesion molecules expression preventing hepatic cells against death in ischemia/reperfusion injury [75].

Bilirubin exerts antiproliferative properties by interfering with pro-carcinogenic signaling pathways, such as the effect on ERK1/2 [78]. Likewise bilirubin, BVR was proved to decrease the expression and phosphorylation of oncogenic factors including Akt pathway [79]. In another study, the antiproliferative effects of bilirubin were evaluated in suppressing the development of vascular hyperplasia, and thus bilirubin seems to be a promising protective agent in vascular diseases [80].

1.1.3.3 Ferrous ion/Ferritin

Generally, ferrous iron is known for its cytotoxic effects even in low concentrations, producing hydroxyl radicals in organisms. However, the spectrum of actions for this small ion is wider. Heme catabolism-generated ferrous iron, being an oxidant, stimulates the synthesis of ferritin through binding to its regulatory protein and activation of iron response elements [81].

Ferritin concentration was proved to be elevated following *HMOX1* induction and decreased with *HMOX* inhibition [82], and it was recognized for its anti-oxidative properties. Mammalian ferritin is composed of two subunit types, the H- and L-chains. H-ferritin contains the ferroxidase center, which is the catalytic site accelerating Fe(II)

oxidation, a rate-limiting step in the mechanism of ferritin iron incorporation. H-ferritin regulates the metabolic iron pool with a mechanism dependent on the functionality of ferroxidase centers, which results in reduce in proliferation and increase in the resistance to oxidative stress [83]. H-ferritin induced by NF-κB has been shown to suppress ROS accumulation by sequestrating iron. By this mechanism, JNK signaling is inhibited resulting in the decrease of TNF-induced apoptosis [84]. Interestingly, H-ferritin can translocate to the nuclei in some cell types to protect DNA from iron toxicity, or can be actively secreted [85]. This can enhance the antioxidant capacity of the heme catabolic pathway.

Besides the ferrous ion scavenging capacity, intracellular ferritin has been recently shown to have non-iron mediated roles *in vivo*. For example, ferritin has been reported to associate with apolipoprotein B suggesting the connection of iron to cholesterol metabolism [86]. Iron regulatory pool may play an important role in regulating folate metabolism and thereby thymidine biosynthesis via induction of serine hydroxymethyltransferase [87]. Furthermore, cytosolic H ferritin is involved in chemokine receptor signaling and receptor-mediated cell migration [88].

1.2 Hepatic bile acid and bile pigment transport

1.2.1 Bile acid transport

Bile acids (BAs) represent a group of acid steroids with remarkable range of physical, chemical and biological effects in organism. Biosynthesis of BAs represents a process by which hydrophobic insoluble cholesterol is converted into the amphipathic molecules of BAs. This formation includes a series of enzymatic reactions localized in cytosol, microsomes, mitochondria, and peroxisomes to produce cholic acid (CA) and chenodeoxycholic acid (CDCA), the primary BAs in human. The presence of BAs in organism is usually limited to the enterohepatic circulation that includes the liver, bile ducts, intestine and portal blood [89]. Since the majority of the circulating BAs are conjugated to taurine or glycine amino acids in the form of membrane impermeable anions (in physiological pH), the transcellular transport of BAs involves specialized transporters in both hepatocytes and enterocytes [90].

Under normal physiological conditions, BAs in hepatocytes are immediately transported to the canalicular membrane to be secreted to bile. However, during pathological conditions, i.e. cholestasis, BAs can be transported back to blood. This process of BAs efflux across the basolateral membrane of the hepatocytes is mediated by members of the multidrug resistance-associated proteins (MRPs), namely MRP3 (*ABCC3*, OMIM*604323) and MRP4 (*ABCC4*, OMIM*605250) [91].

The secretion of BAs across the canalicular membrane represents the rate-limiting step in hepatic secretion and bile formation [91]. The major transporters involved in mediating hepatic BAs secretion include bile salt export pump (BSEP, *ABCB11,* OMIM*603201) that is responsible for transport of monovalent BAs, and MRP2 that is the main transporter of divalent sulfated or glucuronosylated BA (reviewed in [92]).

The intestinal transport is the second important component of BAs enterohepatic circulation since the majority of both conjugated and unconjugated BAs are efficiently absorbed from the intestinal lumen back into blood. The unconjugated BAs can be rapidly transported into enterocytes via passive diffusion along the intestine whereas conjugated BAs require for absorption Na+-dependent mechanism. In distal ileum, BAs are actively absorbed via Na+-dependent apical sodium-dependent bile acid transporter (ASBT, ISBT, *SLC10A2*, OMIM*601295) into enterocyte facilitated by ileal bile acid binding protein (IBABP).

Subsequently, BAs are secreted through organic solute transporter α and β (OSTA, *SLC51A*, OMIM*612084; OSTB, *SLC51B,* OMIM*612085) into portal blood, the majority of BAs reach the space of Disse through the large fenestrae of the sinusoids as albumin-bound complexes. After the contact of the BA (complex albumin-BA) with the basolateral membrane of hepatocyte, conformational changes in albumin structure lead to BA release. The transport of BAs is managed via several transporters on the basolateral (sinusoidal) membrane of hepatocytes, utilizing mainly two processes: Na+-dependent and Na+-independent uptake. Na+-dependent uptake activity in the hepatocytes has been shown to be predominantly mediated by the Na+-dependent taurocholic cotransporting polypeptide (NTCP, *SLC10A1,* OMIM*182396). NTCP has been shown to mediate the transport of conjugated and unconjugated, as well as sulfated BAs [93]. Microsomal epoxide hydrolase (EPHX, *EPHX1,* OMIM*132810) and organic anion transporting polypeptides (OATPs) also transport BAs on sinusoidal membrane in a Na+-dependent and Na+-independent manner, respectively. The OATP family (*SLCO*, previously referred as *SLC21*) includes several members involved in Na+-independent BAs uptake, predominantly OATP1A2 (*SLCO1A2*, previously known as OATP-A, OMIM*602883), OATP1B1 (*SLCO1B1*, previously referred as OATP-

C/OATP2, OMIM*604843) and OATP1B3 (*SLCO1B3*, previously referred as OATP8, OMIM*605495). Interestingly, BAs appear to reduce the expression of several OATP transporters. Thereby the enterohepatic circulation is completed (reviewed in [92]) (Fig.4).

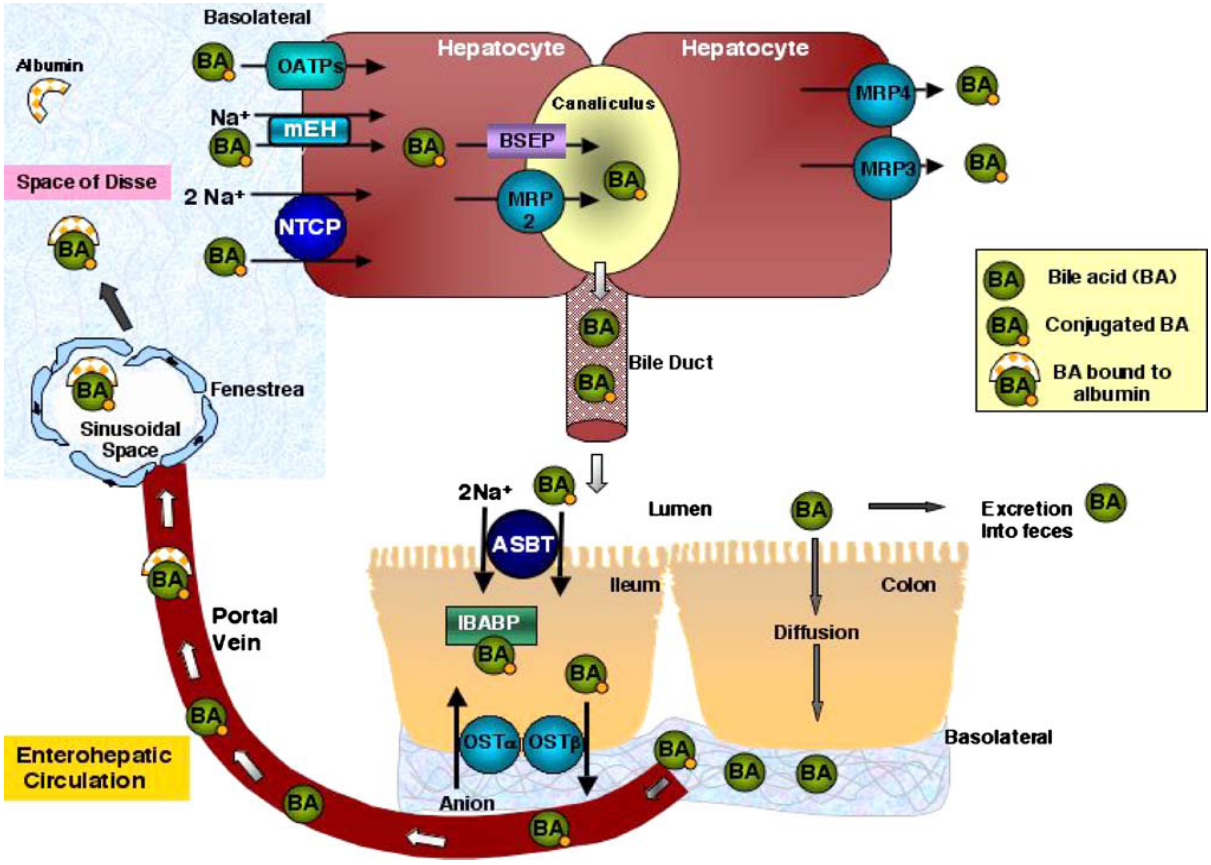

Fig.4. Enterohepatic circulation of bile acids. *BAs transport across the basolateral membrane of the hepatocytes is mainly mediated by NTCP. EPHX (mEH) and OATPs also transport BAs in a Na+-dependent and Na+-independent manner, respectively. BAs efflux across the basolateral membrane of hepatocytes occurs via MRP3 and MRP4. The secretion of BAs across the canalicular membrane occurs via two members of the ATP-binding cassette transporters BSEP that transports monovalent BAs, and MRP2 that transports divalent sulfated or glucuronosylated BAs. BAs are delivered to the intestinal lumen through the bile duct where they participate in emulsifying dietary lipids. Some of the secreted BAs are deconjugated in the intestinal lumen by the action of the bacterial flora. The majority of both conjugated and unconjugated BAs are absorbed from the intestinal lumen and only about 5 % get secreted. While unconjugated BAs may passively diffuse across the small intestinal and colonic epithelia, BAs are actively absorbed in the distal ileum via ASBT. The intracellular transport of BAs across the enterocytes is facilitated by IBABP while they efflux through OSTα /OSTβ. BAs re-enter the portal blood completing their enterohepatic circulation ([92], with kind permission of Springer Science+Business Media).*

1.2.2 Bilirubin transport

Bilirubin released from heme degradation enters the blood circulation as unconjugated bilirubin (UCB) and circulates largely bound to albumin. The circulation of UCB in the bloodstream terminates after it reaches the hepatocyte and is transferred via basolateral membrane into ER. Inside the hepatocyte, UCB is conjugated with glucuronic acid, and subsequently, conjugated bilirubin is secreted into bile. Following deconjugation and series of reduction/oxidation reactions by the intestinal flora, urobilinogens and urobilins are secreted in the stool. However, a proportion of unconjugated bilirubin may be reabsorbed from the gut lumen under specific conditions.

The exact mechanism of UCB uptake is still unknown and both passive diffusion and active transport via several sinusoidal transporters have been proposed. The ability of UCB to enter any cell when the plasma concentration reaches a certain threshold supports the spontaneous diffusion via basolateral hepatocyte membrane as the main mechanism. Nevertheless, during last 20 years at least four putative transporters for transmembrane passage were suggested, including bilirubin/BSP-biding protein, organic anion-binding protein, bilitranslocase, and OATP, however, the confirmatory evidence for their role in bilirubin transport is still lacking. New data indicate that two members of OATP family, namely OATP1B1 and OATP1B3, may mediate hepatic bilirubin transport. Once transmitted into hepatocyte, UCB is transported within the cell bound to a group of cytosolic proteins, preferentially glutathione S-transferase B (ligandin or protein Y) and fatty acid-binding protein 1 (FABP1 or protein Z). Part of the UCB pool comprises of membrane-bound UCB. In ER, UCB is conjugated with glucuronic acid by UGT1A1 (reviewed in [94]).

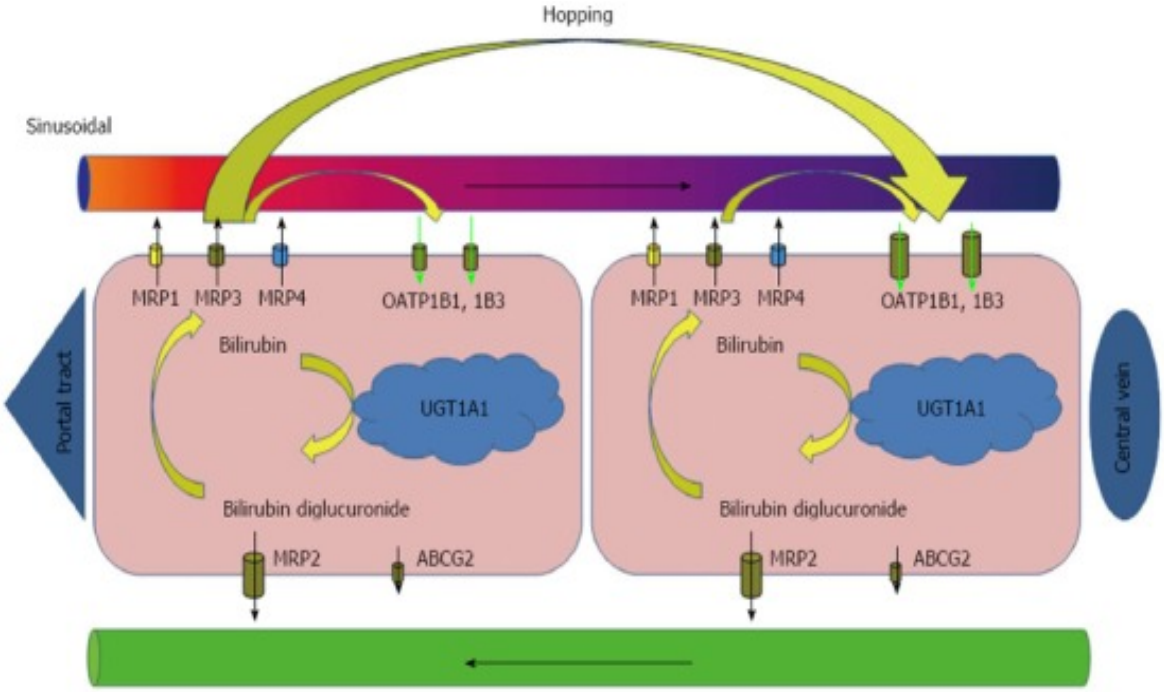

Fig.5. Intrahepatic cycle of conjugated bilirubin. *Bilirubin conjugated in ER of hepatocytes is secreted into bile. This process is mediated by MRP2 with possible minor contribution of other transporters (ABCG2) at the canalicular membrane of hepatocytes. In addition, even under physiologic conditions, a fraction of bilirubin conjugates is secreted by MRP3 across the sinusoidal membrane into blood, from where they can be subsequently reuptaken by sinusoidal membrane-bound OATP1B1 and OATP1B3 transporters of centrilobular hepatocytes where is found the highest expression of OATP1Bs. The process of substrate shifting (hopping) from periportal to centrizonal hepatocytes may act as a protection of the periportal hepatocytes against elevated concentrations of various xenobiotics ([95], with permission of Baishideng Publishing Group Inc.).*

The secretion of conjugated bilirubin into bile is mediated by an ATP-dependent MRP2 and, to a lesser extent, also by ATP-binding cassette (ABC) efflux transporter (*ABCG2*, OMIM*603756). To protect the hepatocyte against high concentration of conjugated bilirubin in the absence or impaired function of MRP2, bilirubin flow may be redirected into sinusoidal blood via MRP3 transporter (reviewed in [94]).

Interestingly, recent studies have shown that a significant fraction of conjugated bilirubin is secreted into the sinusoidal blood and subsequently reuptaken by hepatocytes under physiological conditions in the process that is mediated by sinusoidal transporters MRP3, OATP1B1 and OATP1B3 [96]. This „hepatocyte hopping“ might protect periportal hepatocyte against oversaturation of canalicular secretion and elevated concentrations of various xenobiotics (Fig.5) [94, 95].

1.3 Pathogenesis of selected liver diseases

1.3.1 Cholestasis

The liver is a unique organ where a complex of specialized functions takes place, including metabolism of proteins, carbohydrates and lipids, *de novo* synthesis of BAs and cholesterol, and elimination of toxic waste products into bile.

In the intestinal lumen, BAs are essential for the absorption of lipids, cholesterol and lipid-soluble vitamins. The diversity in hydrophilic/hydrophobic characters of BAs may contribute to BAs interactions with other substances, including changes in solubility of phospholipids, cholesterol, and other lipids [97]. BAs regulate the intestinal uptake of specific minerals [98], and display endocrine function [99]. Among others, BAs have antimicrobial effects [100]. Recently, BAs were revealed as very important signaling molecules in the liver and gastrointestinal tract [101].

The disruption in balanced bile salts metabolism is linked to various defects leading to the liver diseases, including cholestasis. Cholestasis may result from an impairment of bile formation, secretion or/and flow. The reduction in hepatic water and/or organic anion secretion (e.g. bilirubin, BAs) appears. These organic anions that are normally secreted into bile are retained in blood. Cholestasis is typically characterized by an elevation in serum alkaline phosphatase activities, bilirubin and BAs circulation, and if progressive, fibrosis, cirrhosis and clinical signs of liver failure may ultimately develop [102].

Cholestasis is classified as extra- and intrahepatic, and can be acute or chronic. Extrahepatic cholestasis is caused by an obstruction within the biliary tree. The most common causes of obstructive cholestasis are concrements in common bile duct or cancer of bile duct system and/or pancreas. In contrast, intrahepatic cholestasis is

caused by hepatic transporter impairment, exposure to medicaments, sequels of hepatitis, hormonal dysbalance, primary biliary cirrhosis or sepsis [102].

Estrogens were linked to cholestasis in susceptible women during pregnancy, administration of oral contraceptives or hormone replacement therapy [103]. Estrogens have been shown to target the biliary tree, where they modulate the proliferative and secretory activities of cholangiocytes, the epithelial cells lining bile ducts [104]. The administration of ethinylestradiol (EE) to rats reduced bile flow and the biliary secretion of organic anions, such as bilirubin and bile salts, without changes of the hepatic morphology [105]. However in animal models, elevated levels of estrogens and progesterone in plasma contribute to important changes in major hepatic transporters expression and regulation representing the model of intrahepatic cholestasis in man [106].

The mechanism of hepatocellular damage in cholestasis is not clear but seems to be connected to the accumulation of toxic substances, especially BAs. High concentration of BAs leads to free radicals production and a series of consequent reactions resulting in apoptosis and cell necrosis [107]. Several animal models of cholestasis that resemble cholestatic liver injury in man have facilitated studies of the pathophysiology of cholestasis. Other mechanism which may also contribute to cholestasis include disruption of the cytoskeleton and vesicle transport, impairment of signal transduction pathways, defects in tight junctional structures, and the destruction of bile ductules/ducts by immunological or toxic mechanisms [108].

1.3.2 Liver and inflammation

While the metabolic and secretory function of the liver is primarily dependent on liver parenchymal cells, i.e. hepatocytes (HCs), nonparenchymal cell populations such as liver sinusoidal endothelial cells (SEc), hepatic stellate cells (HSc) and Kupffer cells (KCs) display various functions including the role in liver growth and regeneration [109], and immune defense [110]. The liver actively modulates inflammatory processes by filtrating, inactivating, and clearing bacteria, bacterial products, vasoactive substances, and inflammatory mediators [111]. Thus the loss of liver functions represents a serious consequence of inflammation.

The liver represents one of the first defense systems against microorganisms and microbial products (e.g. LPS), in particular those originating from the gut lumen. For specific recognition of bacterial products, pattern-recognition receptors (PRRs) are used [112]. Among PRRs, Toll-like receptors (TLRs) are widely expressed on parenchymal and non-parenchymal cells in the mammalian liver, and are able to recognize pathogen-associated molecular patterns from microbes and damage-associated molecular patterns from endogenous components of dying host cells [113]. Toll-like receptor 4 (TLR4) in both parenchymal and several non-parenchymal hepatic cells is responsible for LPS recognition. In addition to TLR4, other toll-like receptors such as TLR2, TLR3, and TLR5 appear to be involved in the innate response to microbial infection (bacteria, fungi, viruses) [114].

KCs are the first to encounter LPS originating from the gut lumen. LPS in the bloodstream is bound to LPS binding protein (LBP) forming a complex with a soluble or membrane-bound CD14 [115] to bind to TLR4 on the plasma membrane of cells in the liver. Activated TLR4 initiates the myeloid differentiation factor 88 (MyD88)-dependent pathway activation of IL-1 receptor-associated kinase (IRAK), resulting in an

activation of NF-κB and AP-1 in the nucleus. Subsequently, pro-inflammatory cytokines (TNFα, IL-1β, IL-6, IL-8, chemokines) are released and bound to their respective receptors in the hepatocytes [116] (Fig.6). In addition to pro-inflammatory cytokine release, KCs produce anti-inflammatory cytokine IL-10 following stimulation with LPS [117].

The release of pro-inflammatory cytokines triggers various cascades of inflammatory processes which, if not regulated, lead to liver injury. TNFα is recognized as the initial and important mediator of many inflammatory processes. It mediates production of acute phase proteins by the liver and has been shown to induce apoptosis. IL-1 binds to the receptor on monocytes, neutrophils and hepatocytes, and stimulates production of pro-inflammatory enzymes, such as cyclooxygenases, tissue-degrading proteases, various adhesion molecules and NO. IL-6 is involved in the production of acute phase proteins by hepatocyte. IL-8 acts as a chemokine that stimulates hepatic transmigration of neutrophils [116]. KCs-derived IL-12 and IL-18 activate hepatic natural killer cells to increase the synthesis of antimicrobial IFNγ [118]. On the contrary, LPS-stimulated KCs produce IL-10 which contributes to downregulation of pro-inflammatory cytokines [119]. Besides KCs, also HCs, HSc and SEc can produce cytokines [120, 121].

In hepatocytes, inflammatory signals attenuate the expression of hepatocellular transporters both in the canalicular and basolateral membranes [122, 123]. Downregulation of hepatic transport systems affecting BA uptake and secretion as well as downregulation of phase I and phase II detoxification systems result in impairment in bile formation, and accumulation of BAs and toxins in the liver and serum [124]. Thus, inflammation-induced cholestasis becomes an integrated response to inflammatory signals of acute-phase response.

Pathological conditions accompanied by cholestasis and inflammation comprise primary sclerosing cholangitis (PSC), autoimmune hepatitis, sepsis or posttransplant rejection [125]. Chronic liver damage from excessive inflammation signals might result in the development of fibrosis-associated hepatocellular carcinoma (HCC) [126].

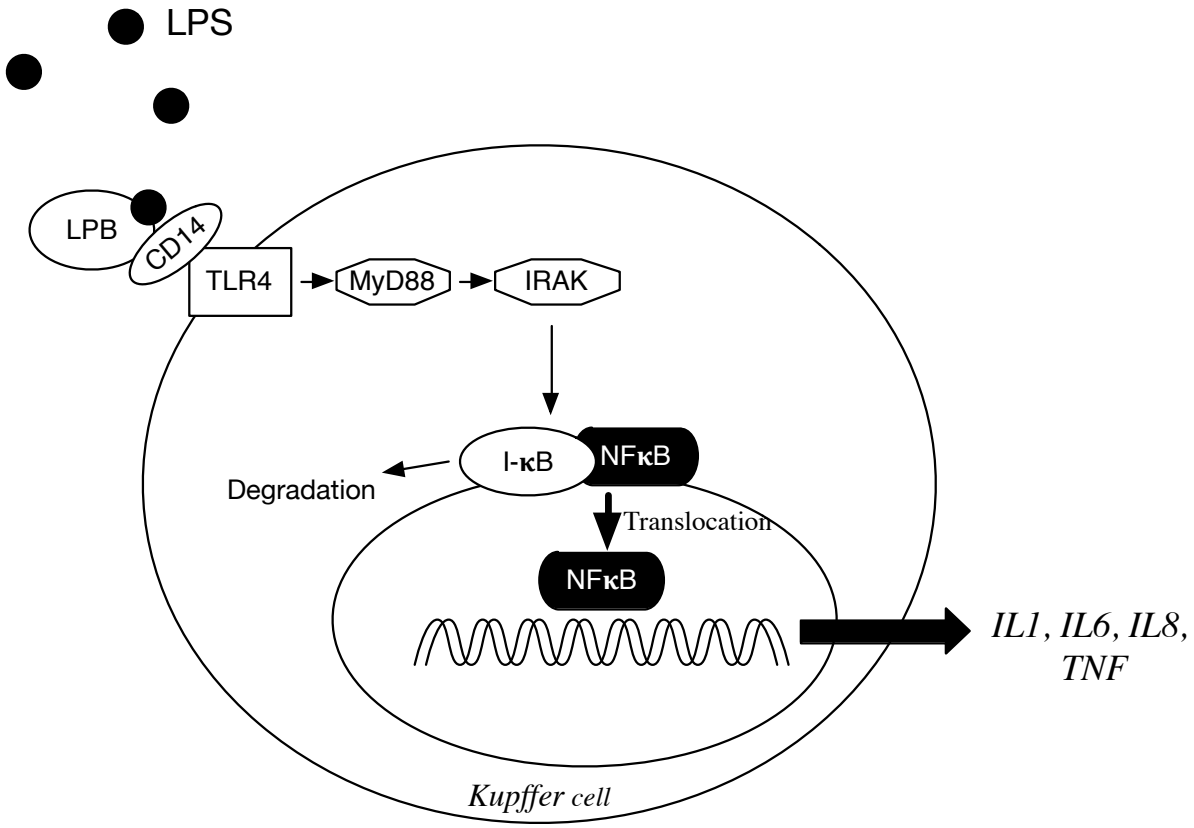

Fig.6. LPS-mediated production of inflammatory cytokines in Kupffer cells through activation of the nuclear transcription factor κB. *LPS from Gram-negative bacteria bound to LPB forms complex with CD14. Through TLR4 this complex initiates a MyD88-dependent pathway, including activation of IRAK. Inhibitor protein (I-κB) is degraded which allows NF-κB to translocate to the nucleus, resulting in transcription of inflammatory cytokines. Upregulation of pro-inflammatory cytokines may result in hepatocyte injury.*

1.4 Heme catabolic pathway in liver diseases

During last decades, the regulation of many hepatobiliary functions by the heme catabolic pathway has been described and thus the heme degrading system might play an important role in the pathogenesis of liver diseases.

The role of the heme catabolic pathway in inflammation has been described, including direct actions in the liver [127-129]. Induction of *HMOX1* in hepatocytes protected against TNF-mediated apoptotic cell death and subsequent neutrophil-induced injury to hepatocytes during endotoxemia [128]. Also, CO was shown to protect against LPS-induced hepatic injury by inhibiting cytokine production and reduction of LPS-induced iNOS expression and nitrite accumulation [129]. Both *hmox1* induction and exogenous CO administration protected mice from apoptotic liver damage caused by cytokines [127] .

Using CORM-2, exogenous CO reduced the extent of apoptosis and pro-inflammatory stress response in the model of hepatic ischemia reperfusion injury that is characterized by hepatocellular death and inflammatory cell influx [130]. Additionally, beneficial effects of induction of *HMOX1* were shown in ischemia/reperfusion injury in steatotic livers [131].

Several studies suggest the role of CO in the maintenance of bile flow and liver integrity during cholestatic processes. Suematsu et al. found that the Hmox activity inhibitor zinc protoporphyrin IX eliminates endogenous CO flux evoking sinusoidal constriction in a perfused rat liver [132]. The same Hmox inhibitor increased BA-dependent bile output accompanying an increased secretion of bile salts suggesting the active role of CO in the regulation of biliary function [133]. Furthermore, CO is recognized as a regulator of bile canalicular contractility [134]. A recent study illustrated that both overproduction of CO by the *hmox1* induction and exogenous CO

administration stimulates BA-independent choleresis while suppressing biliary secretion of bile salts, indicating the stimulation of fluid secretion into bile [42]. CO has been shown to upregulate *Abcc2* and thus enhance biliary output by increasing the biliary secretion of glutathione [135]. Last but not least, bilirubin plays an important role in the hepatocyte protection against oxidative stress that is increased cholestasis [107]. The protective effect of bilirubin is connected to its capability for radicals scavenging and lipid and lipoproteins peroxidation suppression [136].

Induction of *HMOX1* expression was identified to exert chemoprotective effects against hepatocarcinogenesis. HMOX1 was found to inhibit human HCC cell migration and tumor growth by suppressing the expression of *IL6* both *in vitro* and *in vivo* [137]. Interestingly, *HMOX1* overexpression has been observed to decrease hepatitis C virus replication, reduce pro-oxidant production in replicon cells, and increase resistance to oxidative injury [138]. Furthermore, induction of *HMOX1* protects against liver injury in the acute hepatitis B model [139].

Collectively, these data demonstrate the importance of the heme catabolic pathway in the protection of liver integrity via several mechanisms including anti-apoptotic, chemopreventive, antiproliferative, vasoactive, anti-oxidative and anti-inflammatory effects (Fig.7). However, the data on the role of the *HMOX1* induction and its catabolic products in cholestatic disease and associated complications are lacking.

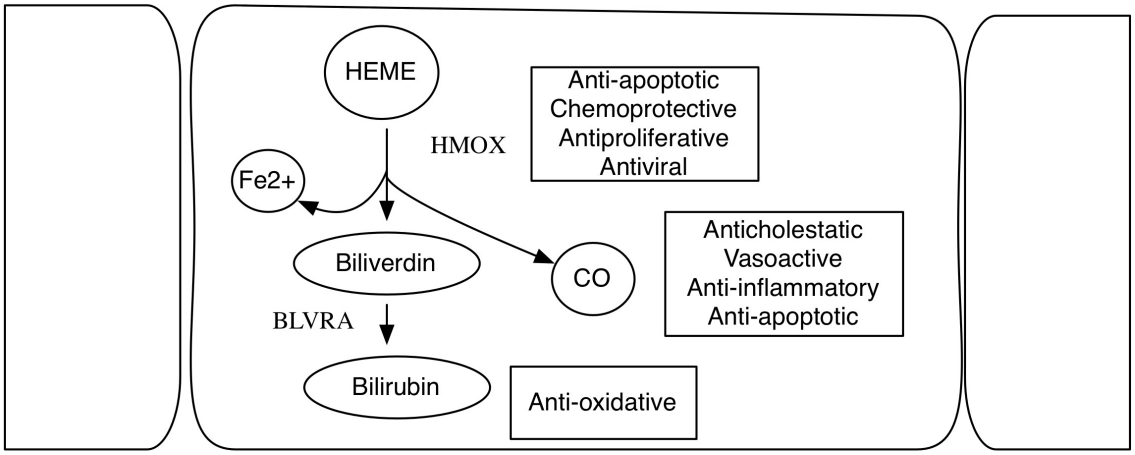

Fig.7. Effects of heme catabolic pathway on hepatocytes. *Induction of HMOX1 and its catabolic pathway was connected to various protective effects in the liver diseases through several signaling pathways.*

2 AIMS

The aim of this study was to clarify the role of Hmox and its metabolic products in the pathogenesis of selected liver diseases.

1. Hmox1 was previously shown to act as a hepatoprotective agent in endotoxin-induced liver injury. One of the Hmox1 inducers, curcumin, possesses anti-inflammatory and antioxidant properties. Whether curcumin exerts its inducing effects on Hmox1 under both physiological and stress conditions in the endotoxin/D-galactosamine model of liver injury has not been described yet. **Thus, the aim of our study was to elucidate the effect of curcumin on Hmox1/CO antioxidant system under oxidative stress condition in the liver** (*reported in „Hepatoprotective effect of curcumin in lipopolysaccharide/D-galactosamine model of liver injury in rats: Relationship to HO-1/CO antioxidant system"* [140]).

2. The Hmox1/CO system displays anti-inflammatory, antiproliferative, antioxidant and hepatoprotective properties. Moreover, CO has been shown to be an important factor in maintaining the balance between liver sinusoidal perfusion and biliary transport, affecting bile canalicular contractility. **We were interested if this unique combination of anti-inflammatory and anticholestatic actions of CO might be useful in the treatment of endotoxin-induced cholestasis where the bile flow is impaired by inflammatory cytokines released by endotoxin** (*results reported in „Protective effects of inhaled carbon monoxide in endotoxin-induced cholestasis is dependent on its kinetics"* [141]).

3. CO inhalation has been evaluated as a potential treatment strategy for several diseases. However, the capability of extragenously administered CO to reach target

organs and its subsequent elimination ratio *in vivo* has not been evaluated so far. **Another aim of our study was to clarify whether inhaled carbon monoxide can reach the specific organs to exert its biological effects** (results in „*Protective effects of inhaled carbon monoxide in endotoxin-induced cholestasis is dependent on its kinetics"* [141]).

4. Estrogens were identified as another cause of intrahepatic cholestasis, a rather frequent liver disease in susceptible women during pregnancy or after hormonal therapy. Estrogens have been shown to alter hepatic transporter expression on both sinusoidal and canalicular membranes, since the treatment of this condition is currently only symptomatic, **we were interested if the Hmox induction can prevent estrogen-induced cholestasis in rats and to identify the possible mechanism of this regulation** (results in „*Protective effect of heme oxygenase induction in ethinylestradiol-induced cholestasis*" [142]).

5. BAs carry out several important functions in the organism affecting lipid metabolism, enzyme secretion, antimicrobial defense and nuclear factor targeting. However, BA accumulation in hepatocytes during obstructive cholestasis is the major cause of cholestatic liver injury. BAs impair mitochondrial respiration and stimulate the generation of ROS leading to oxidative stress damages. Interestingly bilirubin, another marker of cholestasis, displays strong antioxidant properties both *in vitro* and *in vivo* studies. **Thus, we aimed to elucidate the relation between these two major cholestatic markers regarding to their role in oxidative stress during cholestasis** (*reported in „Bile acids decrease intracellular bilirubin levels in the cholestatic liver: implications for bile acid-mediated oxidative stress"* [143]).

6. Accumulating evidence indicates that bilirubin has a beneficial role as an antioxidant. In our previous work we have shown that the intracellular unconjugated bilirubin can be depleted in oxidative stress-related diseases leading to the reduction of cell antioxidant capacity. Highly differentiated distribution of unconjugated bilirubin in various tissues correlated to the serum levels was reported but the mechanism regulating intracellular bilirubin has not been described. **The aim of our study was to explain intracellular metabolism and antioxidant properties of bilirubin under stress conditions *in vitro* as well as *in vivo* in the endotoxin-induced liver injury** (*reported in „Intracellular accumulation of bilirubin as a defense mechanism against increased oxidative stress"* [144]).

3 PUBLICATIONS

3.1 Cerny D, Lekic N, Vanova K, Muchova L, Horinek A, Kmonickova E, Zidek Z, Kamenikova L, Farghali H. (2011) Hepatoprotective effect of curcumin in lipopolysaccharide/D-galactosamine model of liver injury in rats: Relationship to HO-1/CO antioxidant system. Fitoterapia 82(5), 786-791.

3.2 Muchova L, Vanova K, Zelenka J, Lenicek M, Petr T, Vejrazka M, Sticova E, Vreman HJ, Wong RJ, Vitek L. (2011) Bile acids decrease intracellular bilirubin levels in the cholestatic liver: implications for bile acid-mediated oxidative stress. J Cell Mol Med. 15(5), 1156-1165.

3.3 Zelenka J, Muchova L, Zelenkova M, Vanova K, Vreman HJ, Wong RJ, Vitek L. (2012) Intracellular Accumulation of Bilirubin as a Defense Mechanism Against Increased Oxidative Stress. Biochimie 94(8), 1821-1827.

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Hepatoprotective effect of curcumin in lipopolysaccharide/D-galactosamine model of liver injury in rats: Relationship to HO-1/CO antioxidant system

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ABSTRACT

This work studied a relationship between HO-1/CO system and lipid peroxidation with consequent effects on liver functions and NOS-2. We focused on curcumin pretreatment in rat toxic model of D-galactosamine and lipopolysaccharide. Hepatocyte viability, lipid peroxidation, antioxidant status, ALT and AST were evaluated. HO-1 and NOS-2 expressions and respective enzyme activity were determined. Curcumin caused decreases in ALT and AST levels as well as in lipid peroxidation. Furthermore, curcumin pretreatment increased liver HO-1 (2.4-fold, $p = 0.001$), but reduced NOS-2 (4.1-fold, $p = 0.01$) expressions. In conclusion, the tuning of CO/NO pathways is important in shedding light on curcumin's cytoprotective effects in this model.

1. Introduction

Curcumin is a yellow pigment from rhizomatous herbaceous plant turmeric (*Curcuma longa*). This low-molecular-weight polyphenol exhibits reported anti-inflammatory [1] and antioxidant [2] properties. It is generally regarded as the most active constituent of the rhizomes and comprises 2–8% of most turmeric preparations. It has long been used as the yellow spice in Indian food and as a herbal medicine for the treatment of inflammatory diseases [3]. Several reports are dealing with curcumin as a potential cytoprotective agent [4–11]. Due to its antioxidant and anti-inflammatory properties, curcumin is considered as an hepatoprotective and anti-fibrotic compound [12,13]. Curcumin is also known as an inducer of enzyme heme oxygenase-1 by several mechanisms [14].

Heme oxygenase-1 (HO-1), an inducible enzyme in heme catabolic pathway, has emerged as an important mediator of antiinflammatory, antioxidant and cytoprotective actions. HO-1 and its metabolic products, CO and bilirubin, are potential drug targets for amelioration of liver insults [15]. Molecular targets of CO are heme proteins, including among several others nitric oxide synthases (NOSs). Nitric oxide produced in high amounts by NOS-2 greatly contributes to the pro-oxidative status of the cells and can also stimulate HO-1 [16]. Various dose combinations of LPS and DG were used to produce sub lethal liver failure which simulate clinical situations in viral, drug or alcohol-induced, immune-induced or under ischemia reperfusion hepatitis [17–22]. While LPS causes cytokine release and increase in reactive oxygen/nitrogen species, D-GalN inhibits protein synthesis due to depletion of the uridine triphosphate pool in addition to its well established pro-oxidative effect [23].

The aim of this study was therefore to evaluate whether curcumin, as an antioxidant and cytoprotective substance, exerts its inducing effects on HO-1 under normal physiological conditions as well as in response to stress conditions in

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the present liver failure model. The specific goal of the work was directed to study the mutual relationship between HO-1/CO system and lipid peroxidation with consequent effects on liver functions and inducible NOS-2/NO system.

2. Materials and methods

2.1. Materials

Lipopolysaccharide from *Escherichia coli* K-235 (LPS), D-galactosamine hydrochloride (DG) FW 179,17, curcumin (CUR) FW 368.38 - purity ≥ 80,1% curcumin, 16% other curcuminoids (demethoxycurcumin$^+$ bisdemethoxycurcumin) and the rest 3,9% is not defined, TRIS–HCl, EDTA sodium, sucrose, trichloracetic acid (TCA), ammonium molybdate tetrahydrate, hydrogen peroxide, heptane, isopropyl alcohol, potassium phosphate, nicotinamide adenine dinucleotide phosphate (NADPH), hemin, bovine serum albumin (BSA), sodium phosphate Na_3PO_4, anhydrous magnesium perchlorate and sulphosalicylic acid were obtained from Sigma-Aldrich (Prague, Czech Republic). Carbon monoxide as calibration gas for gas chromatography was purchased from Linde (Prague, Czech Republic). Bio-Rad protein assay dye reagent concentrate was obtained from Bio-Rad Company (Prague, Czech Republic). A reagent kit Cayman Chemical Company (An Arbor, MI) for NO_2^-/NO_3^- measurement. The universal kit GeneAmp® RNA PCR using murine leukemia virus (MuLv) reverse transcriptase (RT) and TagMan® Gene Expression Assays Kit for heme oxygenase-1, nitric oxide synthase-2, beta-2-microglobulin including probes, primers, $MgCl_2$, dNTP mixture, 10x PCR buffer and DNA-polymerase were obtained from Applied Biosystems (Prague, Czech Republic). Quiagen RNeasy Plus Mini Kit for total RNA isolation was obtained from Bio-Consult Laboratories (Prague, Czech Republic).

2.2. Animals and drug treatments

Male Wistar rats of 200–300 g body weight (Velaz-Lysolaje, Czech Republic) were used throughout the present study and were allowed water and standard granulated diet *ad libitum*. All rats received humane care in accordance with the general guidelines of the First Faculty of Medicine, Charles University in Prague. The study protocol was approved by the Faculty Ethical Committee.

2.2.1. Drug treatments

We used a dose of 400 mg/kg DG *i.p.* followed immediately by 10 μg/kg LPS *i.p*. At the beginning of our experiment, two models using two different concentrations of LPS, 10 μg/kg and 50 μg/kg, were used. Only the first one (10 μg/kg) has been chosen for our further studies, because it produced reversible fulminant hepatic failure which was suitable for the present study. According to several reported preclinical studies [24,25], we used an adequate dose of CUR 100 mg/kg *i.p.* 1 h before the hepatic injury by DG + LPS. 24 h later animals were sacrificed by decapitation and blood samples were collected. Plasma was immediately isolated by centrifugation at 805 ×*g* for 10 min and used for assessment of ALT, AST, total bilirubin, catalase and nitric oxide (NO) as NO_2^-. Meanwhile, liver samples were snap frozen in liquid nitrogen and stored at −80 °C for RT PCR and biochemical studies as explained further. In addition, liver homogenates were used for conjugated dienes, total carbon monoxide (CO) and HO-activity measurements.

2.3. Determination of plasma alanine aminotransferase (ALT), aspartate aminotransferase (AST), total bilirubin and NO_2^-

The hepatocyte membrane integrity was assessed from plasma ALT and AST concentrations. Determination of plasma alanine aminotransferase (ALT) was carried out using Fluitest® GPT ALT kit by Analyticon (Germany). The principle of this test is to catalyze a reaction with pyruvate as one of the end products. Pyruvate reacts in the next reaction that involves oxidation of NADH to NAD. The rate of decrease of NADH is measured photometrically and is directly proportional to the rate of formation of pyruvate, which is indicative of ALT activity.

Fluitest® GOT AST kit by Analyticon (Germany) was used in determination of aspartate aminotransferase (AST) plasma levels. Briefly, AST is an enzyme that catalyzes conversion of 2-oxoglutarate into oxalacetate, which further reacts with NADH. The rate of decrease of NADH is measured photometrically and is directly proportional to the rate of formation of oxaloacetate, which in turn is indicative of AST activity.

Total bilirubin in plasma was measured using Fluitest® BIL-Total kit (Czech Republic). This method involves photometric measurement of azobilirubin dye that is produced when bilirubin reacts with sulfanilic acid.

Assessment of plasma NO_2^- was carried out using a colorimetric kit of Cayman Chemical Company (USA) and a microplate reader according to manufacturer's instructions. Briefly, this method involves a conversion of nitrate (NO_3^-) to nitrite (NO_2^-) by nitrate reductase. This is followed by the addition of the Griess reagent (1% sulfanilamide, 0.1% naphtylethylendiamine, 2.5% H_3PO_4) that allows for a simple spectrophotometrical measurement (at 540 nm) of NO oxidation product NO_2^- without the inclusion of nitrate reductase. The NO_2^- levels were calculated by comparison with a $NaNO_2$ standard curve.

2.4. HO-activity and total tissue carbon monoxide measurement

Liver HO activity and tissue CO content were measured as previously described [26]. Briefly, for HO activity measurement, liver homogenates were incubated with methemalbumin (1.5 mM heme/0.15 mM albumin) and NADPH at 37 °C in CO-free septum-sealed vials for 15 min and reaction was terminated with the addition of 60% sulphosalicylic acid. For liver CO determination, liver homogenates were incubated in CO-free septum-sealed vials containing 60% (w/v) sulphosalicylic acid on ice. The amount of CO generated by the reaction and released into the vial headspace was quantitated by gas chromatography with a reduction gas analyzer (Trace Analytical, Menlo Park, CA, USA). HO activity and liver CO content were calculated as pmolCO/hr/mg protein and pmolCO/mg protein, respectively.

2.5. Assay of enzymatic antioxidant (catalase) and lipid peroxidation (conjugated dienes) markers

The catalase assay in liver tissue or plasma was performed spectrophotometrically based on the reaction between H_2O_2 and molybdenium ammonium as previously reported [27]. The results were expressed in μg/ml. Conjugated dienes (CD)

Table 1
Effect of CUR pretreatment in lipopolysaccharide-induced hepatitis in D-galactosamine sensitized rats *on plasma alanine aminotransferase (ALT), aspartate aminotransferase (AST) and bilirubin*; CONTROL — vehicle only; CUR curcumin 100 mg/kg; DG + LPS — D-galactosamine 400 mg/kg + lipopolysaccharide 10 μg/kg after 24 hour incubation; CUR + DG + LPS — curcumin 1-hour pretreatment before D-galactosamine and lipopolysaccharide toxic insult; means ± SEM, n = 7.

	CONTROL	DG + LPS	CUR	CUR + DG + LPS
ALT [μcat/l	27.77 ± 3.02	247.31 ± 54.79 ***	35.89 ± 4.79	113.05 ± 31.92 #
AST [μcat/l]	56.80 ± 4.48	127.87 ± 10.63 ***	57.90 ± 9.79	63.70 ± 9.39 ###
Bilirubin [μmol/l]	3,6748 ± 1,7752	19,3777 ± 3,4768 ***	4,8779 ± 0,8157	36,8939 ± 2,9646 ###

*** Value significant compared to respective group with vehicle only (CONTROL) $p<0.001$.
# Value significant to respective positive control group (DG + LPS) $p<0.05$.
Value significant to respective positive control group (DG + LPS) $p<0.001$.

as lipid peroxidation marker was assayed as described earlier [28]. The results were expressed in nmol/mg protein.

2.6. Expression of inducible genes for HO-1 and NOS-2-real time PCR

Twenty-four hours following drug administration, the liver samples were obtained to be used for total RNA isolation according to the manufacturers' instructions of the Qiagen® RNeasy plus kit. Following total RNA isolation, the reverse transcription from total RNA to cDNA was processed by universal kit GeneAmp® RNA using a murine leukemia virus (MuLv) reverse transcriptase (RT). Real-time PCR was performed using Taqman Gene Expression Assay Kit (Applied Biosystems, USA). Overall three genes were evaluated — HO-1, NOS-2 genes as target genes and beta-2-microglobulin as a control (endogenous or house-keeping) gene. House-keeping gene-expression was stable and constant during the experiment and was used in comparison with target gene-expression. Real-time quantitative PCR analysis was performed by use of ABI PRISM 7900 machine.

2.7. Statistical analysis

The data were expressed as mean ± SEM. All experiments performed in groups of rats resulted in reported means of 7 animals. There were four independent groups together labeled as: CONTROL (negative control group with saline treatment only), CUR (curcumin *per se*), DG + LPS (D-galactosamine and lipopolysaccharide fixed toxic combination; positive control group) and CUR + DG + LPS (group with toxic combination pretreated by curcumin). In each case, there was one independent measurement of each parameter from each rat (24 h after drug application). Data passed normality test, that's why the statistical significance of difference of mean scores was determined using one-way Analysis of Variance (ANOVA) with *post hoc* Bonferroni multiple comparisons test for comparing plasma, tissue and/or genetic alterations between treated (positive control) and intact (negative control) animals.

P-value less than 0.05 was considered statistically significant.

3. Results

3.1. Effect of curcumin pretreatment on liver functions

Significant increases in serum aminotransferases ALT, AST and plasma bilirubin (Table 1) levels were observed in DG + LPS animals reflecting severe hepatocellular injury in this group of animals as compared to rats with saline treatment (negative controls). CUR *per se* had no effect on these parameters. Importantly, pretreatment with CUR resulted in significant drop of ALT and AST in CUR + DG + LPS rats ($p<0.05$), while bilirubin levels further increased (Table 1).

3.2. Effect of curcumin pretreatment on HO activity, HO-1 gene expression and total carbon monoxide tissue content

Discrepancy between increasing plasma bilirubin levels and decreasing aminotransferase activities prompted us to measure the rate of bilirubin production in the liver of experimental animals. DG + LPS toxic combination resulted in significant 5-fold increase in HO-1 mRNA gene expression ($p=0.05$, Fig. 1). Furthermore, curcumin pretreatment was associated with almost 12-fold increase in HO-1 mRNA in CUR + DG + LPS group compared to vehicle-treated controls ($p<0.001$, Fig. 1). The similar increasing trend has been observed for total HO activity (Fig. 2) indicating increased bilirubin production in curcumin pretreated animals with toxic liver injury.

Surprisingly, carbon monoxide, another product of HO reaction, significantly increased in the liver of DG + LPS group (2.5-fold, $p<0.01$), but not in CUR + DG + LPS animals (Fig. 3). Interestingly, curcumin administration to animals without liver injury (CUR group) had no significant effect on

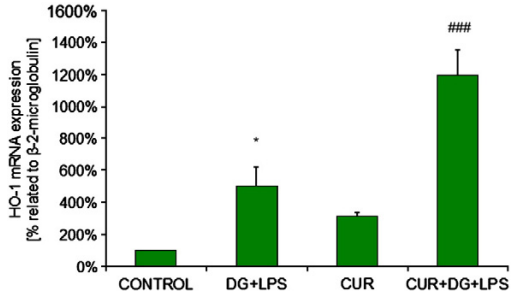

Fig. 1. *Effect of CUR* pretreatment in lipopolysaccharide-induced hepatitis in D-galactosamine sensitized rats *on HO-1 gene expression* related to beta-2-microglobulin as the endogenous control; CONTROL — vehicle only; CUR curcumin 100 mg/kg; DG + LPS — D-galactosamine 400 mg/kg + lipopolysaccharide 10 μg/kg after 24 hour incubation; CUR + DG + LPS — curcumin 1-hour pretreatment before D-galactosamine and lipopolysaccharide toxic insult; * value significant compared to negative control group (CONTROL) $p\leq0.05$, ### value significant to positive control group (DG + LPS) $p\leq0.001$; Means ± SEM, n = 7.

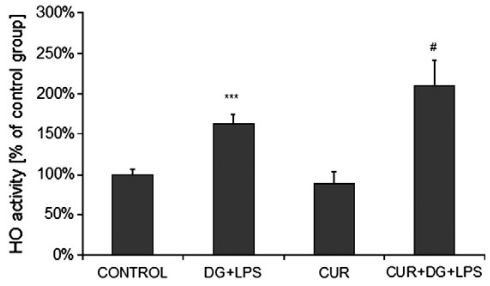

Fig. 2. Effect of CUR pretreatment in lipopolysaccharide-induced hepatitis in D-galactosamine sensitized rats *on total HO activity*; CONTROL — vehicle only; CUR curcumin 100 mg/kg; DG + LPS — D-galactosamine 400 mg/kg + lipopolysaccharide 10 μg/kg after 24 hour incubation; CUR + DG + LPS — curcumin 1-hour pretreatment before D-galactosamine and lipopolysaccharide toxic insult; *** value significant compared to group with vehicle only (CONTROL) $p<0.001$; # value significant to positive control group (DG + LPS) $p<0.05$; Means ± SEM, n = 7.

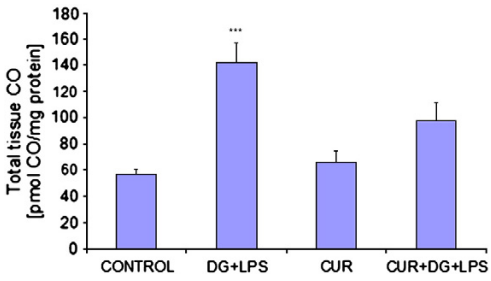

Fig. 3. *Effect of CUR* pretreatment in lipopolysaccharide-induced hepatitis in D-galactosamine sensitized rats *on total tissue carbon monoxide (CO)*; CONTROL — vehicle only; CUR curcumin 100 mg/kg; DG + LPS — D-galactosamine 400 mg/kg + lipopolysaccharide 10 μg/kg after 24 hour incubation; CUR + DG + LPS — curcumin 1-hour pretreatment before D-galactosamine and lipopolysaccharide toxic insult; *** value significant compared to group with vehicle only (CONTROL) $p<0.001$; Means ± SEM, n = 7.

either HO gene expression/activity or CO/bilirubin levels (Figs. 1–3, Table 1).

Since CO is not only the product of heme catabolic pathway, but also one of the products of lipid peroxidation, we decided to measure the extent of lipid peroxidation and oxidative stress markers.

3.3. Effect of curcumin pretreatment on lipid peroxidation, oxidative stress markers and NOS-2/NO system

The extent of lipid peroxidation as evidenced by conjugated diene formation significantly increased in the livers of DG + LPS animals (3.5-fold, $p<0.001$ compared to controls). Curcumin pretreatment reduced the conjugated dienes levels to 1.6-fold of controls in CUR + DG + LPS ($p<0.01$, Table 2).

Interestingly, plasma catalase activity as a marker of antioxidant status was 2.4-fold elevated compared to controls in DG + LPS group and this level further increased to 3-fold following CUR pretreatment (CUR + DG + LPS group) clearly replicating plasma bilirubin concentration (Table 2).

Moreover, it was found that DG + LPS injection produced significantly more liver tissue NO_2^- as compared to control animals (Fig. 4). CUR was able to reduce DG + LPS-induced increase in NO_2^- production at statistically relevant level ($p<0.05$). However, single CUR injection did not affect NO_2^- production in liver of experimental animals. In accordance with these results, DG + LPS *per se* increased NOS-2 mRNA expression (240-fold) in the liver homogenates and CUR pretreatment highly significantly reduced this increase ($p<0.01$), as shown in Fig. 5.

4. Discussion

This work presents considerable hepatoprotective effect of curcumin in DG + LPS liver toxicity model mediated *via* modulation of HO/NOS systems.

Curcumin's cytoprotective, anti-inflammatory, antitumor, and antioxidant properties are based on several mechanisms. The reported ones are mainly based on the suppression of pro-inflammatory mediators like cyclooxygenase-2 (COX-2), lipooxygenase (LOX), inducible nitric oxide synthase (iNOS or NOS-2), nuclear factor kappa B (NF-κB) [29], tumor necrosis factor alpha (TNF-α) and others [30]. Another group of cellular constituents that can be affected by curcumin is the family of heat shock proteins. It was reported that induction of HSP 70 has cytoprotective effects [31], inhibition of HSP 90 results in the enhancement of anti-proliferative and pro-apoptotic activities [32] and induction of HSP 32 (= heme oxygenase-1) is associated with protection against oxidative stress [33].

The toxic combination of DG + LPS resembles clinical hepatitis [34–38]. In this model, we demonstrated ameliorative effects of curcumin pretreatment associated with regulation of HO-1. The liver protection was clearly demonstrated by the significant decrease in serum aminotransferases (ALT, AST) after CUR pretreatment in CUR-DG-LPS rats.

Table 2
Effect of CUR pretreatment in lipopolysaccharide-induced hepatitis in D-galactosamine sensitized rats *on catalase in plasma (CAT) and conjugated dienes in liver homogenate (CD)*; CONTROL — vehicle only; CUR curcumin 100 mg/kg; DG + LPS — D-galactosamine 400 mg/kg + lipopolysaccharide 10 μg/kg after 24 hour incubation; CUR + DG + LPS — curcumin 1-hour pretreatment before D-galactosamine and lipopolysaccharide toxic insult; means ± SEM, n = 7.

	CONTROL	DG + LPS	CUR	CUR + DG + LPS
CAT [μg/ml]	55.43 ± 6.45	132.93 ± 10.20***	41.22 ± 3.23	164.35 ± 9.11#
CD [nmol/mg/protein]	2.19 ± 0.37	7.76 ± 1.49***	1.77 ± 0.42	3.43 ± 0.60##

*** Value significant compared to respective group with vehicle only (CONTROL) $p<0.001$.
# Value significant to respective positive control group (DG + LPS) $p<0.05$.
Value significant to respective positive control group (DG + LPS) $p<0.01$.

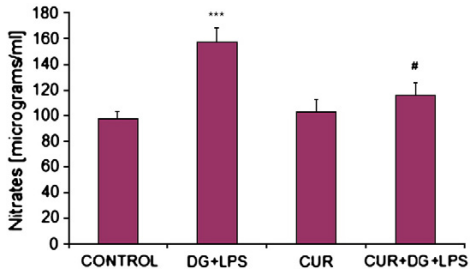

Fig. 4. *Effect of CUR* pretreatment in lipopolysaccharide-induced hepatitis in D-galactosamine sensitized rats *on cellular nitrates* (NO_2^-); CONTROL — vehicle only; CUR curcumin 100 mg/kg; DG + LPS — D-galactosamine 400 mg/kg + lipopolysaccharide 10 μg/kg after 24 hour incubation; CUR + DG + LPS — curcumin 1-hour pretreatment before D-galactosamine and lipopolysaccharide toxic insult; *** value significant compared to group with vehicle only (CONTROL) $p<0.001$, # value significant to positive control group (DG + LPS) $p<0.05$; Means ± SEM, $n=7$.

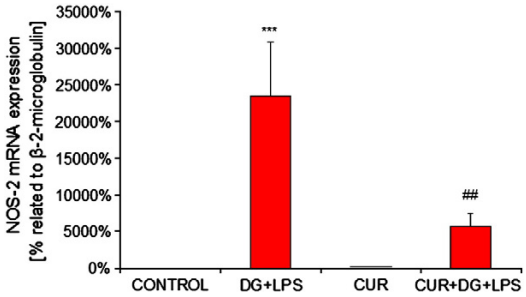

Fig. 5. *Effect of CUR* pretreatment in lipopolysaccharide-induced hepatitis in D-galactosamine sensitized rats *on NOS-2 gene expression* related to beta-2-microglobulin as the endogenous control; CONTROL — vehicle only; CUR curcumin 100 mg/kg; DG + LPS — D-galactosamine 400 mg/kg + lipopolysaccharide 10 μg/kg after 24 hour incubation; CUR + DG + LPS — curcumin 1-hour pretreatment before D-galactosamine and lipopolysaccharide toxic insult; *** value significant compared to negative control group (CONTROL) $p\leq0.001$, ## value significant to positive control group (DG + LPS) $p\leq0.01$; Means ± SEM, $n=7$.

Interestingly, plasma bilirubin levels further increased in this group of animals. We demonstrate that this increase is not attributed to the severity of liver injury, but rather to the increased bilirubin production represented by concomitant elevation of HO activity and expression. This observation is of significant importance as bilirubin has been reported to possess strong antioxidant properties and to protect liver from oxidative stress-mediated injury [39,40]. Given its hydrophobic properties, unconjugated bilirubin has been shown to protect organism primarily against lipid peroxidation [41]. However, similar effect has been described for water-soluble conjugated bilirubin or albumin-bound bilirubin *in vitro* [42] and *in vivo* [43].

In this study, DG + LPS administration resulted in dramatic increase in lipid peroxidation as measured by conjugated dienes and nitrate production and NOS-2 mRNA expression in the liver and/or plasma of experimental animals. Curcumin pretreatment caused significant drop in all these parameters. Furthermore, increased catalase activity in plasma of curcumin pretreated rats reflects the increased antioxidant status of experimental animals under current pathophysiological conditions. Our results are in accordance with published data showing antioxidant and anti-inflammatory effects of curcumin in various models of organ injury [44–46].

Furthermore, our finding of HO-1/NOS system modulation is reflected in *in vitro* studies on RAW264.7 macrophages suggesting that inhibitory effect of curcumin on NO production is mediated via HO-1 up-regulation inactivating nuclear factor (NF)-kappa B [47].

Moreover, the decrease of lipid peroxidation after curcumin pretreatment might explain slightly lower level of liver CO after curcumin pretreatment despite higher HO activity. It has been published that CO is not only the product of heme catabolic pathway catalyzed by heme oxygenase, but also one of the products of lipid peroxidation [43,48]. Thus, lipid peroxidation might significantly influence final tissue pool of CO.

Importantly, single application of curcumin without concomitant oxidative stress and inflammatory liver injury had no effect either on HO-1 activity and expression or on NOS-2/NO system and oxidative status *in vivo*. We hypothesize that different regulation mechanisms might be involved under physiological and/or pathophysiological conditions as suggested in previous *in vitro* studies [14,49].

We conclude that this hepatoprotective effect of curcumin in LPS + DG model of liver injury is associated with significant up-regulation of HO-1 and/or its antioxidant and cytoprotective product bilirubin. This effect is followed by down-regulation of NOS-2 expression and consequent NO production and lower accumulation of lipid peroxidation products in liver and/or plasma of experimental animals.

Acknowledgments

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Bile acids decrease intracellular bilirubin levels in the cholestatic liver: implications for bile acid-mediated oxidative stress

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Abstract

High plasma concentrations of bile acids (BA) and bilirubin are hallmarks of cholestasis. BA are implicated in the pathogenesis of cholestatic liver damage through mechanisms involving oxidative stress, whereas bilirubin is a strong antioxidant. We evaluated the roles of bilirubin and BA on mediating oxidative stress in rats following bile duct ligation (BDL). Adult female Wistar and Gunn rats intraperitoneally anaesthetized with ketamine and xylazine underwent BDL or sham operation. Cholestatic markers, antioxidant capacity, lipid peroxidation and heme oxygenase (HO) activity were determined in plasma and/or liver tissue 5 days after surgery. HepG2-rNtcp cells were used for *in vitro* experiments. Plasma bilirubin levels in control and BDL animals positively correlated with plasma antioxidant capacity. Peroxyl radical scavenging capacity was significantly higher in the plasma of BDL Wistar rats (210 $\pm$ 12%, $P < 0.0001$) compared to controls, but not in the liver tissues. Furthermore after BDL, lipid peroxidation in the livers increased (179 $\pm$ 37%, $P < 0.01$), whereas liver HO activity significantly decreased to 61% of control levels ($P < 0.001$). Addition of taurocholic acid (TCA, $\geq$50 μmol/l) to liver homogenates increased lipid peroxidation ($P < 0.01$) in Wistar, but not in Gunn rats or after the addition of bilirubin. In HepG2-rNtcp cells, TCA decreased both HO activity and intracellular bilirubin levels. We conclude that even though plasma bilirubin is a marker of cholestasis and hepatocyte dysfunction, it is also an endogenous antioxidant, which may counteract the pro-oxidative effects of BA in circulation. However, in an animal model of obstructive cholestasis, we found that BA compromise intracellular bilirubin levels making hepatocytes more susceptible to oxidative damage.

Keywords: taurocholic acid • heme oxygenase • carbon monoxide • lipid peroxidation

Introduction

Obstructive cholestasis, characterized by a failure to secrete bile into the bile duct and intestine, results in the accumulation of bile acids (BA) and bilirubin in circulation. Elevated activities of cholestatic enzymes and plasma levels of bilirubin and BA are used as laboratory markers of cholestasis.

The accumulation of BA inside hepatocytes is the major cause of cholestatic liver damage [1], including structural and functional injuries of hepatocyte membranes [2], cell death [3] and activation of inflammatory and fibrogenic signalling pathways [4]. Several studies have suggested an important role of increased oxidative stress in the pathogenesis of cholestatic injury [5, 6]. Accumulated BA within hepatocytes impair mitochondrial respiration and electron transport and stimulate the generation of reactive oxygen species (ROS) in hepatic mitochondria [7]. Accordingly, mitochondrial free radicals may then modify nucleic acids, proteins and lipids. In fact, an increase in lipid peroxidative products has been observed in cholestatic livers [8].

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The liver possesses a complex defence system including antioxidant enzymes and substrates to control the formation of ROS and repair oxidative damage [9]. Bilirubin, a product of heme catabolism, is a potent antioxidant substance both *in vitro* [10] and *in vivo* [11]. *In vitro* studies with liposomes have shown that both unconjugated (UCB) and conjugated bilirubin (CB) are protective against lipid peroxidation, surpassing that of α-tocopherol, an important lipid-soluble antioxidant [10]. Antioxidant properties of bilirubin were further confirmed by a number of animal and clinical studies demonstrating the protective effects of bilirubin on the development of atherosclerosis [12–14], cancer [15, 16] and other oxidative stress-mediated diseases [17].

The objective of this study was to address the seemingly dichotomous effects of high levels of the antioxidant bilirubin and the pro-oxidant BA in obstructive cholestasis using an animal model.

Materials and methods

Animals

Female Wistar rats obtained from Anlab (Prague, Czech Republic) and hyperbilirubinemic Gunn rats (RHA/jj, in-house colony from 1st Faculty of Medicine, Charles University in Prague) with a congenital deficiency of bilirubin uridine 5′-diphospho (UDP)-glucuronosyltransferase, both weighing from 200 to 280 g, were provided water and food *ad libitum*. All aspects of the animal studies met the accepted criteria for the care and experimental use of laboratory animals, and all protocols were approved by the Animal Research Committee of the 1st Faculty of Medicine, Charles University in Prague.

Reagents

L-Ascorbic acid, 2,6-di-*tert*-butyl-4-methylphenol (BHT), bovine serum albumin (BSA) 98%, UCB, chloroform (high-performance liquid chromatography [HPLC] grade), hemin, nicotinamide adenine dinucleotide phosphate (NADPH), sulfosalicylic acid, taurocholic acid (TCA) and ethylenediaminetetraacetic acid (EDTA) were purchased from Sigma-Aldrich (St. Louis, MO, USA). All other chemicals were of analytical grade purchased from Penta (Prague, Czech Republic). UCB was purified and recrystallized according to McDonagh and Assisi [18]. Purified UCB was dissolved in 0.1 M NaOH and immediately neutralized with phosphoric acid. The mixture was subsequently diluted with BSA solution to reach a final concentration of 480 μM UCB and 500 μM BSA in phosphate buffer (25 mM, pH 7.0).

Bile duct ligation (BDL)

Rats were anaesthetized with ketamine (90 mg/kg) and xylazine (10 mg/kg) intraperitoneally, and biliary trees were exposed through midline abdominal incisions. Microsurgical ligation of bile ducts and resections of extrahepatic biliary tracts were performed as previously described ($n = 7$ in each group) [19]. Sham-operated (SH) rats underwent the same procedure without bile duct resection and ligation ($n = 6$ in each group).

Tissue preparation

After 5 days, all animals were killed and blood (5 ml) was collected from superior vena cava, transferred to tubes containing EDTA, mixed, and placed on ice. An aliquot was centrifuged to separate plasma. Livers were then harvested, thoroughly washed with 10 ml heparinized saline, and rinsed in ice-cold reaction buffer (0.1 M phosphate buffer, pH 7.4). For RNA analysis, 100 mg of tissue was immediately placed in 1.5 ml microfuge tubes containing RNAlater (Qiagen, Valencia, CA, USA). Tubes were stored at −20°C until total RNA isolation.

For HO activity, HO-1 protein, and lipid peroxidation measurements, 100–150 mg tissue was diluted 1:9 (by weight) in reaction buffer, diced, and sonicated with an ultrasonic cell disruptor (Model XL2000, Misonics, Farmingdale, NY, USA). Sonicates were kept on ice and assayed for HO activity or lipid peroxidation within 1 hr or frozen in liquid nitrogen and stored at −80°C until analysis of HO-1 protein.

For liver carbon monoxide (CO) measurements, 150–200 mg tissue was diluted 1:4 in reaction buffer and then sonicated as described above.

For malondialdehyde (MDA) and 4-hydroxyalkenal analysis, 200 mg of tissue was placed in the Eppendorf tube containing 0.1 M PBS, pH 7.4 with 1% BHT, diced and sonicated. Sonicates were stored at −80°C until analysis.

Markers of cholestasis

Plasma biochemical markers (alkaline phosphatase [ALP], albumin) were determined in an automatic analyser (Hitachi, Model 717, Tokyo, Japan), using standard assays. Total plasma BA levels were determined spectrophotometrically using a Bile Acids kit (Trinity Biotech, Jamestown, NY, USA).

Liver histology

For histological examination, left lateral lobes of livers were fixed overnight in 10% buffered formalin (pH 7.4) at 4°C followed by a standard procedure for paraffin embedding. Serial sections (6 μm thick) were cut and stained with haematoxylin and eosin, Shikata's orcein method, or elastic-van Gieson stain. Each slide was viewed using standard light microscopy.

Peroxyl radical scavenging capacity

Peroxyl radical scavenging capacity was measured fluorometrically as a proportion of chain-breaking antioxidant consumption present in a biologic sample (plasma, liver homogenate) relative to that of Trolox (a reference and calibration antioxidant compound) as previously described [20].

Bilirubin determination

Plasma and liver CB and UCB levels were determined using an HPLC method as previously described [21]. Briefly, pigments were extracted into chloroform-hexane and subsequently delipidated by second extraction into a minute volume of alkaline aqueous solution. The resulting droplet was separated on HPLC.

Heme oxygenase (HO) activity

Twenty microlitres of 10% liver sonicate (2 mg fresh weight [FW]) was incubated for 15 min. at 37°C in CO-free septum-sealed vials containing 20 μl of 150 μM methemalbumin and 20 μl of 4.5 mM NADPH as previously described [22]. Blank reaction vials contained 0.1 M phosphate buffer, pH 7.4, in place of NADPH. Reactions were terminated by adding 5 μl of 30% (w/v) sulfosalicylic acid. The amount of CO generated by the reaction and released into the vial headspace was quantitated by gas chromatography (GC) with a reduction gas analyser (Trace Analytical, Menlo Park, CA, USA). HO activity was calculated as pmol CO/hr/mg FW.

Real-time RT-PCR

Total liver RNA was isolated using phenol : chloroform extraction and cDNA was generated using random hexamer primers and Moloney Murine Leukemia Virus (M-MLV) reverse transcriptase (Promega, Madison, WI, USA). Real-time PCR was performed with TaqMan® Gene Expression Assay Kit for HO-1 (Applera, Alameda, CA, USA). Data were normalized to glyceraldehyde 3-phosphate dehydrogenase and hypoxanthine phosphoribosyl transferase levels, and then expressed as fold change from control.

Western blots

One hundred micrograms of liver sonicates were mixed with equal volume of loading buffer. Samples were separated on 12% polyacrylamide gel and then transferred to polyvinylidene difluoride membranes (Millipore, Bedford, MA, USA). After blocking in Tween-PBS with 5% milk for at least 1 hr, membranes were incubated with HO-1 antibody (1:666; Stressgen, Victoria, BC, USA), or β-actin (1:8000; Sigma-Aldrich) for 1 hr. After washing, membranes were incubated with anti-mouse IgG-HRP (1:2000; Sigma-Aldrich) for 30 min. After washing, immunocomplexes on the membranes were visualized with ECL Western Blotting Detection Reagents (Amersham Biosciences, Buckinghamshire, UK). HO-1 protein bands were quantified by densitometry, normalized to β-actin, and then expressed as fold change from control.

Liver tissue CO

Forty microlitres of liver sonicate was added to CO-free, septum-sealed vials containing 5 μl of 60% (w/v) sulfosalicylic acid. After 30 min. incubation on ice, CO released into the vial headspace was quantitated by GC as previously described [23].

Carbonylhaemoglobin (COHb) determination

Total haemoglobin was estimated to be 15 g/dl for all the animals. COHb was measured by GC as previously described [24] and expressed as percentage of total haemoglobin.

Lipid peroxidation

Twenty microlitres of liver sonicate was incubated for 30 min. at 37°C with 100 μM ascorbate (80 μl) and 6 μM Fe^{2+} (0.5 μl). BHT (100 μM) was added for the blank reaction. CO produced into vial was quantitated by GC as previously described [25]. The amount of CO produced serves as an index of lipid peroxidation and was expressed as pmol CO/hr/mg FW. Total amounts of lipid peroxidation end-products, MDA and 4-hydroxyalkenals were determined using Bioxytech® LPO-586 Assay (Oxis International, Beverly Hills, CA, USA).

Cell culture

The human hepatoblastoma cell line (HepG2) (purchased from American Type Culture Collection, Manassas, VA, USA) and HepG2 cell line stably transfected with Ntcp transporter (HepG2-rNtcp), kindly provided by Professor Ulrich Beuers (University of Munich, Germany; currently University of Amsterdam, Netherlands), were cultured as described previously [26]. Cells were grown on 10 cm Petri dishes (Orange Scientific, Braine-l'Alleud, Belgium), incubated with TCA for 24 hrs for HO activity and intracellular bilirubin or 4 hrs for mRNA determination. After incubation, cells were quickly washed three times with 10 ml PBS, harvested, centrifuged and pellet was dispersed in 300 μl of 0.1 M phosphate buffer, pH 7.4. For mRNA determination, cell pellets were snap frozen in liquid nitrogen and stored at −80°C until analysis. An aliquot of the cell sonicate was used for protein determination (Bio-Rad DC protein assay, Hercules, CA, USA).

Statistical analyses

Normally distributed data are presented as means ± S.D. and analysed by Student t-test. Non-normally distributed datasets are expressed as medians (25%–75%) and analysed by Mann-Whitney rank sum test. The association between plasma bilirubin levels and antioxidant capacity was tested using Spearman rank-order correlation analysis. Differences were deemed statistically significant when $P < 0.05$.

Results

Markers of cholestasis and liver histology

Significant increases in total BA and ALP were observed in all BDL rats (Table 1). As expected, plasma bilirubin levels were significantly elevated in Wistar rats after BDL. In Gunn rats, which are deficient in bilirubin UDP-glucuronosyltransferase, plasma UCB levels remained unchanged after BDL (Table 2), as expected because most of the bilirubin does not enter the intestinal lumen *via* biliary excretion, but rather *via* direct diffusion across the intestinal mucosa [27].

Histological analysis of liver specimens from BDL rats revealed signs of impaired bile flow, such as large bile duct obstruction with intralobular bilirubinostasis (predominantly in perivenular localisation) and biliary interface activity with portal tract oedema, swelling of periportal hepatocytes and marked ductular proliferation with a disruption of the parenchymal limiting plates, accompanied by polymorphonuclear infiltration. Bile plugs

Table 1 Cholestatic markers and liver and body weights

	Wistar (SH) ($n = 6$)	Wistar (BDL) ($n = 7$)	Gunn (SH) ($n = 6$)	Gunn (BDL) ($n = 7$)
Body weight (g)	235 (233–237)	218 (212–240)	253 (206–280)	220 (200–264)
Liver weight (g)	10.4 (9.8–11.0)	14.0 (13.6–14.6)**	11.0 (10.5–11.5)	12.9 (11.5–13.6)*
TBA (μmol/l)	14.5 (10.3–23.3)	326 (290–404)**	8.5 (8.0–12.8)	288 (248–416)**
ALP (μkat/l)	2.1 (1.9–2.2)	4.4 (4.2–4.6)**	0.9 (0.8–1.0)	5.1 (3.9–5.6)**

Cholestatic markers and liver and body weights in SH and BDL Wistar and Gunn rats 5 days after surgery. Data are presented as median (25–75%).
*$P < 0.05$,**$P < 0.001$ compared to corresponding SH group.
TBA: total plasma bile acids, ALP: alkaline phosphatase.

Table 2 Plasma and liver bilirubin

	SH-plasma (μmol/l) ($n = 6$)	BDL-plasma (μmol/l) ($n = 7$)	SH-liver (nmol/g) ($n = 6$)	BDL-liver (nmol/g) ($n = 7$)
Wistar				
TB	0.32 (0.16–0.37)	193.8 (176.4–195.5)**	2.65 (2.33–3.50)	40.7 (31.5–57.1)**
CB	0	181.4 (164.8–190.2)**	1.73 (1.18–2.39)	39.2 (30.1–54.8)**
UCB	0.32 (0.16–0.37)	8.67 (3.75–13.03)**	1.07 (0.88–1.28)	2.03 (1.75–2.37)*
Gunn				
UCB	137.8 (130.0–145.4)	149.1 (135.4–212.6)	45.2 (40.7–46.1)	24.6 (19.6–28.5)*

Plasma and liver bilirubin in SH and BDL Wistar and Gunn rats 5 days after surgery. Data are presented as medians (25–75%).
*$P < 0.05$, **$P < 0.001$, compared to corresponding SH group.
TB: total bilirubin, UCB: unconjugated bilirubin, CB: conjugated bilirubin.

were present in a few cholangioles and bile infarcts were found in periportal zones.

Bilirubin increases antioxidant capacity in plasma, but not in liver homogenates of BDL rats

Peroxyl radical scavenging capacity was significantly higher in BDL compared to SH Wistar rats (210 ± 13 and 100 ± 30%, respectively $P < 0.001$) (Fig. 1A). We suggest that this increase could be attributed to elevated plasma bilirubin levels. In fact, plasma antioxidant capacity correlated positively with plasma total bilirubin levels (Spearman correlation coefficient = 0.45, $P = 0.027$). Unlike in plasma, we did not find any differences in peroxyl radical scavenging capacity in liver homogenates of BDL and SH Wistar rats (113 ± 17 and 100 ± 17%, respectively, $P = 0.21$) (Fig. 1A).

To further confirm our hypothesis, we investigated the effect of bilirubin and that of TCA on peroxyl radical scavenging capacity in normal rat plasma. Addition of bilirubin resulted in a dose-dependent increase in peroxyl radical scavenging capacity, whereas no effect was observed with TCA (Fig. 1B).

Liver bilirubin levels are relatively decreased compared to plasma in BDL animals

Markedly different antioxidant capacities of plasma and liver homogenates of BDL Wistar rats prompted us to measure bilirubin concentrations in those two compartments. Compared to SH rats, plasma bilirubin levels were 606 times higher in BDL Wistar rats. Surprisingly, in liver sonicates, only a 15-fold increase of bilirubin was observed in BDL rats. In Gunn rat livers, we found a significant decrease of 46% in the liver bilirubin levels in BDL rats compared to SH rats, whereas no significant differences were found in plasma (Table 2).

Bilirubin production is decreased and lipid peroxidation is increased following BDL

To identify the possible underlying mechanism for the relative lack of bilirubin in cholestatic hepatocytes, we investigated the rate of bilirubin production in the liver of Wistar rats. Activity of HO, the rate-limiting enzyme of bilirubin synthetic pathway, was

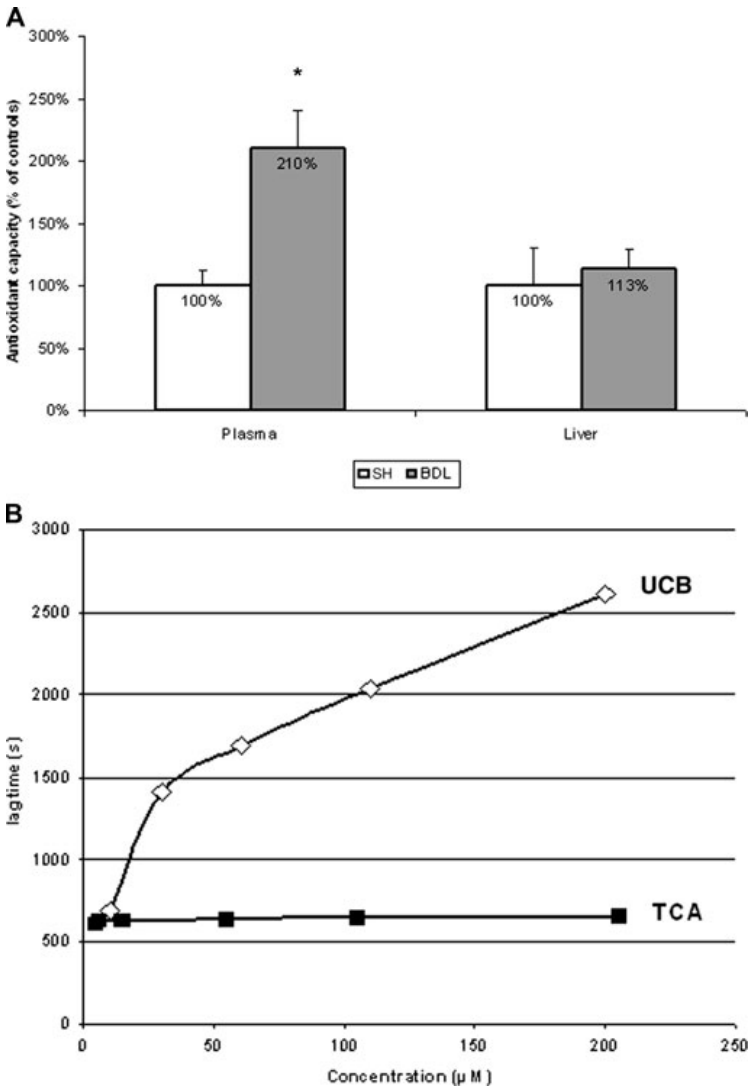

Fig. 1 Antioxidant capacity in plasma and liver homogenates of control (SH) and BDL rats. Effect of bilirubin and TCA. (**A**) Peroxyl radical scavenging capacity of plasma and liver homogenates from SH ($n = 6$) and BDL Wistar rats ($n = 7$). (**B**) Effect of UCB and TCA on peroxyl radical scavenging capacity (lag time) of normal rat plasma.

significantly decreased (54%) in the livers of BDL rats compared to controls (199 ± 36 *versus* 327 ± 48 pmol CO/hr/mg FW, $P = 0.003$). Similarly, HO-1 mRNA expression and HO-1 protein were also significantly down-regulated in BDL livers (Fig. 2A).

Surprisingly, we found significant increases in liver tissue CO and blood COHb levels of cholestatic rats compared to those of controls (7.9 ± 2.4 *versus* 4.0 ± 1.1 pmol CO/mg FW and 0.36 ± 0.04 *versus* 0.18 ± 0.02% total haemoglobin, respectively, $P < 0.05$, Fig. 2B). Because lipid peroxidation represents another source of CO *in vivo* [28], besides HO activity, we analysed the potential for lipid peroxidation. Livers of BDL animals were more susceptible to lipid peroxidation than control livers (85 ± 18 *versus* 47 ± 17 pmol CO/mg FW, respectively, $P = 0.005$). These results were confirmed by direct measurements of liver MDA and 4-hydroxyalkenals, which were significantly higher in BDL compared to SH rats (122 ± 15 and 99 ± 3 μmol/g, respectively, $P = 0.004$).

TCA increases lipid peroxidation in the liver homogenates

To investigate the role of BA in lipid peroxidation, we analysed the effect of increasing concentrations of TCA in normal Wistar

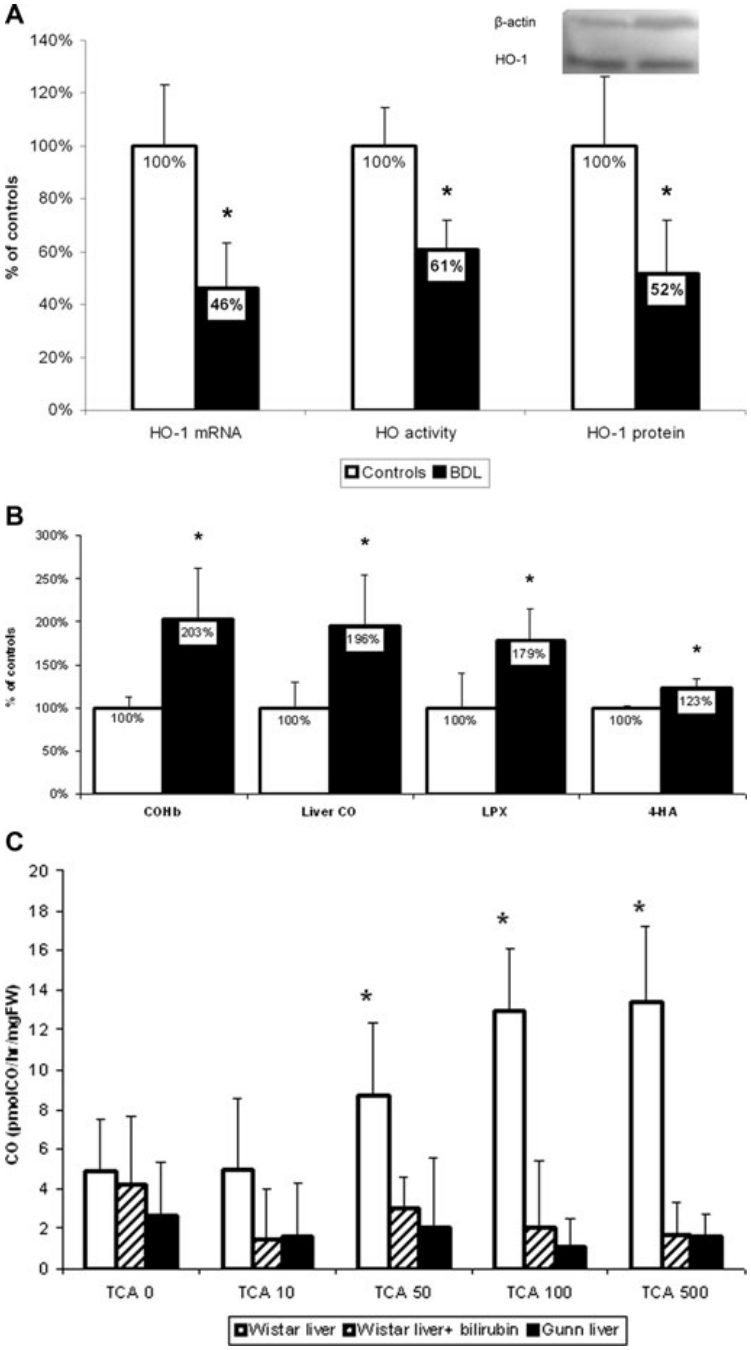

Fig. 2 Bilirubin production and lipid peroxidation following BDL in Wistar rats. Effect of TCA and bilirubin on lipid peroxidation in Wistar and Gunn rat liver homogenates. (**A**) Activity and expression of HO in liver tissue of sham-operated and BDL Wistar rats. Densitometric values of HO-1 protein were normalized to β-actin and all data are expressed as percentage of controls. $^{*}P < 0.05$. (**B**) CO in liver tissue and in the blood (COHb), lipid peroxidation and 4-hydroxyalkenals of cholestatic Wistar rats compared to control animals. Data are expressed as percentage of controls. $^{*}P < 0.05$. (**C**) TCA was added to normal liver homogenates of Wistar and Gunn rats or Wistar rat liver homogenates with 40 μM bilirubin in concentrations of 0, 10, 50, 100 and 500 μM and lipid peroxidation was measured. $^{*}P < 0.05$.

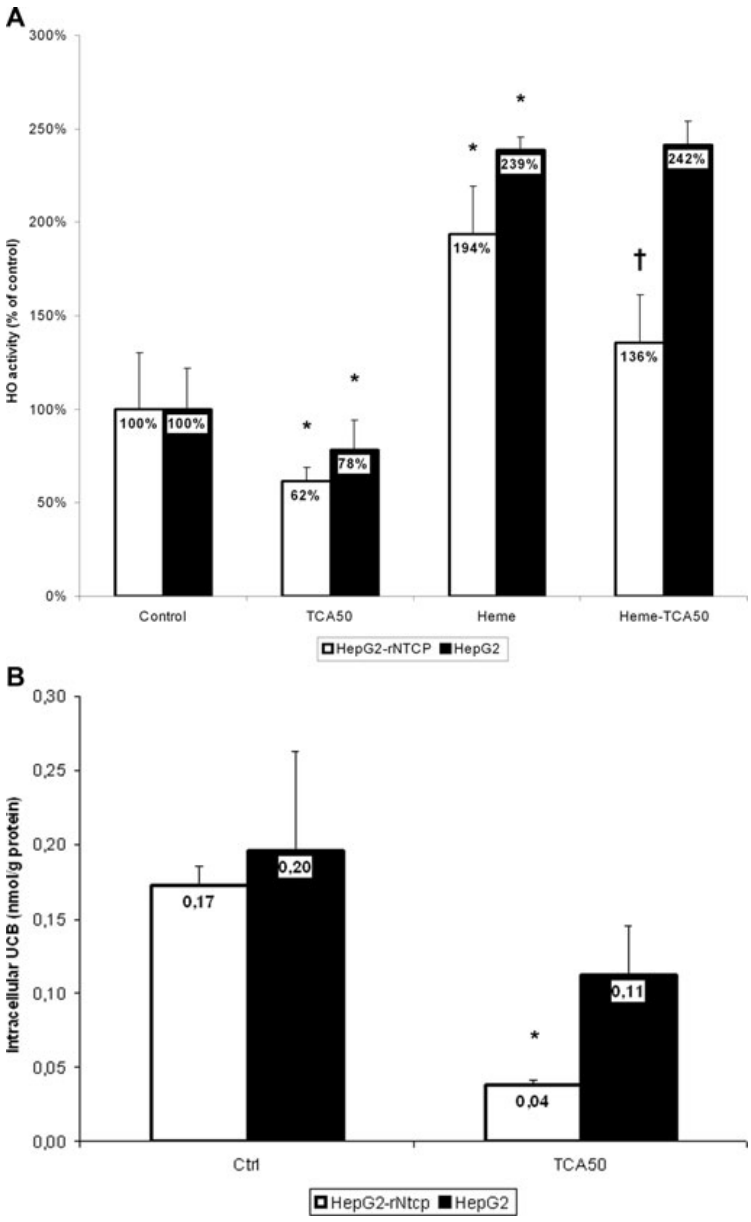

Fig. 3 Effect of TCA on HO activity and intracellular bilirubin *in vitro*. (**A**) HepG2 and HepG2-rNtcp cells (stably transfected with Ntcp transporter) were incubated for 24 hrs with 50 μM TCA, 30 μM heme (HO-1 inducer) or co-incubated with 50 μM TCA and 30 μM heme and HO activity was determined. $^{*}P < 0.05$ compared to controls, $^{\dagger}P < 0.05$ compared to heme-treated cells. (**B**) Intracellular bilirubin levels were measured in HepG2 and HepG2-rNtcp cells 24 hrs after incubation with 50 μM TCA. $^{*}P < 0.05$.

rat and Gunn rat liver homogenates. TCA at concentrations of 50, 100 and 500 μM significantly increased lipid peroxidation in Wistar rat liver homogenates. This effect was completely abolished by addition of 40 μM UCB. In Gunn rats, TCA had no effect on lipid peroxidation within mentioned concentration range (Fig. 2C).

TCA decreases intracellular bilirubin in HepG2-rNtcp cells

Treatment of HepG2 cells stably transfected with the Ntcp transporter with 50 μM TCA resulted in a significant decrease in HO activity (2.43 ± 0.73 nmol CO/hr/mg protein in controls

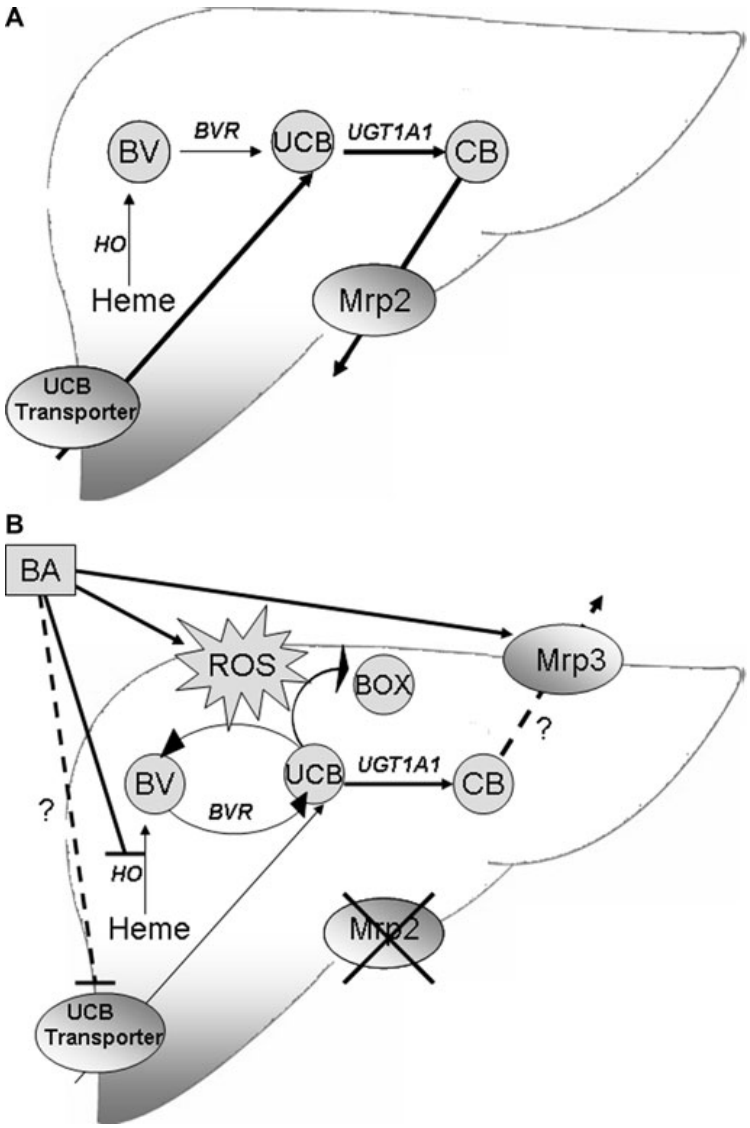

Fig. 4 Proposed bilirubin metabolism in normal (**A**) and cholestatic (**B**) rat liver. (**A**) Under normal conditions, UCB enters hepatocyte *via* carrier mediated mechanism (though the transporter involved still remains to be identified) [36, 37] or is produced intracellulary by oxidative degradation of heme. Intracellular UCB undergoes conjugation catalysed by bilirubin UDP-glucuronosyltransferase (UGT1A1) and CB is eliminated into bile *via* Mrp2 transporter. (**B**) High concentrations of bile acids in cholestatic liver lower bilirubin concentration by (1) triggering oxidative stress which leads to bilirubin consumption through biliverdin reductase (BVR) catalytic cycle [38] and *via* bilirubin oxidation products (BOX) formation (reviewed in [39]), (2) down-regulation of HO resulting in lower bilirubin production and (3) possibly by altering the expression of the basolateral transporters [31]. CB enters systemic circulation across the sinusoidal membrane possibly *via* up-regulated sinusoidal Mrp3 transporter.

versus 1.50 ± 0.11 nmol CO/hr/mg protein in TCA-treated cells, $P = 0.01$, Fig. 3A) and HO-1 mRNA (100% ± 15% *versus* 57% ± 23%, $P = 0.03$). As expected, treatment with 30 μM heme resulted in increase in HO activity though this effect was significantly reduced by co-treatment with TCA. Treatment of HepG2 cells (lacking Ntcp transporter for conjugated BA) with 50 μM TCA resulted in milder decrease in HO activity compared to HepG2-rNtcp cells (1.56 ± 0.34 nmol CO/hr/mg protein in controls *versus* 1.22 ± 0.19 nmol CO/hr/mg protein in TCA-treated cells, $P = 0.03$) and no decrease in HO-1 mRNA (100% ± 10% *versus* 89% ± 13%, $P = 1$). Interestingly, no decrease in HO activity has been observed upon co-treatment with heme plus TCA compared to heme-treated HepG2 cells.

Following treatment with 50 μM TCA, intracellular bilirubin decreased 78% in HepG2-rNtcp cells and only 43% decrease in HepG2 cells compared to control (untreated) cells (Fig. 3B). The decrease of intracellular bilirubin by 78% corresponded to a 31% decrease in HO activity in HepG2-rNtcp cells, suggesting that intracellular bilirubin might be also influenced by other mechanisms (Fig. 4).

Discussion

In this study, we demonstrated that bilirubin is not only a marker of cholestasis and hepatocyte dysfunction; but also, it is an endogenous antioxidant, counteracting the pro-oxidative effects of BA. In addition, we showed that BA lower intrahepatic bilirubin levels and bilirubin production presumably through an interaction between BA and bilirubin.

We found that BDL significantly increases the antioxidative capacity of plasma. Because of the significant positive correlation of plasma antioxidant capacity with bilirubin levels, it appears that bilirubin is the major antioxidant factor. This is supported by the finding that additions of UCB to normal rat plasma increased its antioxidative properties in dose-dependent fashion. These results agree with the data of Granato *et al.* [29] who demonstrated that bilirubin effectively suppresses ROS generation in freshly isolated hepatocytes.

However, a completely different circumstance may exist within liver tissue. BDL did not increase the antioxidant capacity in liver homogenates. We propose that this finding could, at least in part, be explained by the markedly different increases of bilirubin levels in plasma and liver compartments. After BDL, plasma levels of bilirubin increased more than 600-fold; whereas, in the liver only a 15-fold increase was observed. Furthermore, considering the high plasma bilirubin levels, the contamination of the liver with trace amounts of blood could artifactually actually increase liver bilirubin levels. Therefore, liver tissue bilirubin might be even lower in BDL animals compared to controls. Importantly, in Gunn rats, where BDL does not significantly affect plasma bilirubin levels (due to bilirubin elimination across the intestinal mucosa rather than the biliary tract), we observed a marked drop (54%) of liver bilirubin in BDL animals. These findings are of particular importance showing, for the first time, that intracellular bilirubin is actually consumed during cholestasis and that plasma bilirubin concentrations do not necessarily reflect tissue bilirubin metabolism.

To identify possible mechanisms responsible for this lowering of liver bilirubin levels following BDL, we treated HepG2 and HepG2-rNtcp cells with TCA. We found, that TCA down-regulates both the expression and activity of HO (the key enzyme in bilirubin production) and this down-regulation is more pronounced in cells expressing the Ntcp transporter. Accordingly, TCA lowered intracellular bilirubin levels. The markedly higher decrease in intracellular bilirubin compared to that of HO activity suggests that other mechanisms might also be involved. We have previously demonstrated an increased consumption of intracellular bilirubin during oxidative stress [30]. These effects could, together with BA-mediated alteration of bilirubin transport mechanisms [31], account for the relatively low hepatocyte bilirubin levels (Fig. 4).

The present study shows that in obstructive cholestasis, high concentrations of BA are responsible for increased lipid peroxidation in the liver as measured by the accumulation of MDA and 4-hydroxyalkenals, the products of lipid peroxidation. These findings agree with published data showing that MDA levels are increased in the livers of BDL rats [32, 33]. We have also observed increases in liver tissue CO and blood COHb in cholestatic animals. Because HO activity and expression (the main source of CO) are decreased in the livers of BDL animals, our observed elevations of CO concentration could be due to lipid peroxidation [25]. This is supported by our experiments where the addition of TCA to normal liver homogenates increased lipid peroxidation in a dose-dependent manner. However, addition of 40 μM bilirubin to liver homogenate completely abolished this effect. Additionally, no increase in lipid peroxidation was observed following the addition of TCA to liver homogenates from hyperbilirubinemic Gunn rats. All these data further confirm the opposing roles of BA and bilirubin in the development of oxidative stress and support the hypothesis that the higher BA/bilirubin ratio in cholestatic livers could lead to an increased susceptibility of the BDL livers to lipid peroxidation. These observations support also our previous data, showing that treatment of mice with HO inducer, rosuvastatin, led to simultaneous increase in heart HO activity and bilirubin content, but decrease in lipid peroxidation. Pre-treatment with a potent HO inhibitor, tin mesoporphyrin, completely abolished this effect [34].

There are several limitations of our study. We did not measure total BA in cholestatic liver homogenates, however, based on the previous work by Naito *et al.* [35] we can assume that similar concentrations of BA exist both in plasma and the liver. Secondly, only a short-term BDL was performed in our study, therefore, we cannot speculate about the course of chronic cholestasis. To clarify the exact role of HO expression in cholestasis, further studies with HO-1 knockout animals should be performed.

We conclude that high concentrations of BA in cholestasis are responsible for increased lipid peroxidation in the liver. In contrast, bilirubin has an antioxidative effect and is responsible for increased antioxidant capacity of cholestatic plasma. However, in the liver, BA maintain relatively low intracellular bilirubin levels. Therefore, the increase in BA/bilirubin ratio might be implicated in the pathogenesis of oxidative stress-mediated cholestatic liver injury.

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Conflict of interest

The authors confirm that there are no conflicts of interest.

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Intracellular accumulation of bilirubin as a defense mechanism against increased oxidative stress

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ABSTRACT

Antioxidant, anti-inflammatory and anti-atherogenic effects have been associated with elevations of unconjugated bilirubin (UCB) in serum and with the induction of heme oxygenase-1 (HO-1), the rate-limiting enzyme in UCB synthesis. The aim of this study was to investigate the intracellular metabolism and antioxidant properties of UCB in human hepatoblastoma HepG2 cells and tissues of Wistar rats exposed to oxidative stressors and lipopolysaccharide (LPS), respectively. Intracellular UCB concentrations in HepG2 cells correlated with its levels in culture media ($p < 0.001$) and diminished lipid peroxidation in a dose-dependent manner ($p < 0.001$). Moreover, induction of HO-1 with sodium arsenite led to 2.4-fold ($p = 0.01$) accumulation of intracellular UCB over basal level while sodium azide-derived oxidative stress resulted in a 60% drop ($p < 0.001$). This decrease was ameliorated by UCB elevation in media or by simultaneous induction of HO-1. In addition, hyperbilirubinemia and liver HO-1 induction in LPS-treated rats resulted in a 2-fold accumulation of tissue UCB ($p = 0.01$) associated with enhanced protection against lipid peroxidation ($p = 0.02$). In conclusion, hyperbilirubinemia and HO-1 induction associated with inflammation and oxidative stress increase intracellular concentrations of UCB, thus enhancing the protection of cellular lipids against peroxidation. Therefore, the previously reported protective effects of hyperbilirubinemia and HO-1 induction are at least in part due to intracellular accumulation of UCB.

1. Introduction

For decades, unconjugated bilirubin (UCB)[2] has been considered a potentially toxic waste product of heme metabolism. Likewise, systemic hyperbilirubinemia has been generally believed to be an ominous sign of liver disease with a poor prognostic outcome. However, recent studies have demonstrated potent antioxidant and protective effects of UCB, which stand against this paradigm [1–3].

UCB has been found to be an effective lipophilic free radical scavenger [3,4] with significant cytoprotective properties [5]. Antimutagenic [6] and anti-inflammatory [7,8] properties of UCB have also been demonstrated. Several animal studies have shown that hyperbilirubinemia protects against endotoxic shock [8], hypertension [9] and cholestatic liver injury [10]. Moreover, clinical studies also showed that Gilbert's syndrome (mild, chronic, nonhemolytic unconjugated hyperbilirubinemia in the absence of liver disease [2]) is associated with protection against cardiovascular diseases [2,11], cancer [12], and type 2 diabetes [13]. In addition, antioxidant, cytoprotective and anti-inflammatory effects of heme oxygenase (HO) and biliverdin reductase (BVR), the enzymes of UCB production pathway, have been well documented [1,14,15]. Two HO isoforms have been described; HO-1, which is highly inducible by its substrate heme and by various factors associated with oxidative stress (heavy metals, UV light,

Abbreviations: ALP, alkaline phosphatase; ALT, alanine amino transferase; AST, aspartate amino transferase; Bf, unbound fraction of bilirubin; BSA, bovine serum albumin; BVR, biliverdin reductase; DHF, 5-chloromethyl-2′7′-dichlorodihydrofluorescein diacetate acetyl ester; CO, carbon monoxide; DMSO, dimethylsulfoxide; GC-RGD, gas chromatography – reduction gas detector; HO, heme oxygenase; HPLC, high performance liquid chromatography; IM, intramuscular; IP, intraperitoneal; LPS, lipopolysaccharide; MBR, mesobilirubin; MDA, malondialdehyde; MHA, methemalbumin; PBS, phosphate-buffered saline; SnMP, tin mesoporphyrin; TCA, taurocholic acid; UCB, unconjugated bilirubin.

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inflammation), and HO-2, which is constitutively expressed. Protective effects of HO-1 induction are attributed to decreased levels of cellular pro-oxidant heme, increased production of tissue mediator carbon monoxide (CO), and direct modulation of antioxidant genes transcription by HO-1 protein [16]. Whether an intracellular accumulation of UCB is a direct result of HO-1 induction is still under scrutiny.

Taken together, the striking cytoprotective and anti-inflammatory effects of UCB raise the question whether there are mechanisms regulating cellular and tissue UCB levels and, importantly, whether these mechanisms could be pharmacologically targeted. We have recently developed a sensitive HPLC method for determination of UCB in tissues and cells [17]. Using this method, we reported a highly differentiated distribution of UCB in various tissues and described a correlation between UCB levels in rat serum and tissues [17]. Moreover, we found that myocardial UCB levels increase following the pharmacological induction of HO-1 expression [18]. We also demonstrated that following bile duct ligation, UCB levels in rat livers dramatically decreased as a result of increased oxidative stress and decreased liver HO activity [10]. However, detailed studies of the mechanisms regulating intracellular UCB levels are still lacking.

Therefore, in this study, we investigated the antioxidant effects and regulation of UCB metabolism in cells and tissues under various conditions of oxidative stress. We found that HO-1 induction and/or systemic hyperbilirubinemia result in the protective accumulation of UCB in cells and tissues. The accumulation substitutes for consumption of cellular UCB during oxidative stress, thus protecting against excessive lipid peroxidation.

2. Materials and methods

2.1. Chemicals

Heme (in the form of hemin), mesobilirubin (MBR), biliverdin, tin mesoporphyrin (SnMP) and protoporphyrin IXα were purchased from Frontier Scientific (Logan, UT, USA). Dimethylsulfoxide (DMSO) was purchased from Applichem (Darmstadt, Germany). Bovine serum albumin (BSA), UCB, and all the other chemicals and solvents were purchased from Sigma–Aldrich (St. Louis, MO, USA) or as otherwise indicated.

2.2. Preparation of reagents

UCB was purified and recrystalized as described elsewhere [19]. For *in vitro* experiments, UCB was dissolved in 0.1 M NaOH, neutralized with 0.1 M phosphoric acid and immediately stabilized with BSA solution (40 g/L) as described previously [17]. Heme was delivered to cells and animals in the form of methemalbumin (MHA) prepared from hemin and BSA as described previously [20]. Arsenic oxide was converted to $NaAsO_2$ (10 mM) by dissolving in 0.1 M NaOH. SnMP (2.5 mM) was also dissolved in 0.1 M NaOH. Lipopolysaccharide (LPS; 3 mg/mL), taurocholic acid (TCA; 10 mM), and sodium azide (100 mM) were dissolved in saline.

2.3. Cells

In vitro experiments were performed using human HepG2 hepatoblastoma cells (ATCC, Manassas, VA, USA) cultured in Eagle's Minimum Essential Medium (EMEM) with 10% fetal calf serum (FCS). Cells were grown until 70% confluent and then incubated in serum-free media containing 40 g/L BSA with the following compounds (final concentrations in culture media): UCB (0.1–40 μM), heme (30 μM), SnMP (25 μM), TCA (50 μM), $NaAsO_2$ (10–40 μM), or sodium azide (100 μM). After 24 h, plates were washed with phosphate-buffered saline (PBS) and cells collected. Samples were either used fresh (for HO activity and free radicals production) or frozen and stored at −80 °C until analysis. An aliquot of each sample was used for protein determination (DC protein assay, Bio-Rad, Hercules, CA, USA).

2.4. Animals

2.4.1. Experiment I

Adult male Wistar rats ($n = 8$ in each treatment group) were provided water and food *ad libitum*. Animal studies met the accepted criteria for humane care and experimental use of laboratory animals. All protocols were approved by the Animal Research Committee of the 1st Faculty of Medicine, Charles University in Prague. Wistar rats were injected intraperitoneally (IP) with either 6 mg/kg LPS (from *Salmonella typhimurium*) or with saline as a control. After 12 h, animals were anesthetized with intramuscular (IM) injections of ketamine (90 mg/kg) and xylazine (10 mg/kg), and then blood was collected and the circulatory system was perfused with 10 mL of saline containing 500 IU/mL of heparin to remove the blood. Liver, kidney, heart and brain (the latter only from saline treated animals as a matrix for lipid peroxidation determination) were excised and either used fresh or snap frozen in liquid nitrogen, and stored at −80 °C until further use.

2.4.2. Experiment II

Wistar rats ($n = 8$ in each treatment group) were injected IP with either 10 mg/kg of heme or saline (control) at day 1 and day 3. At the day 6, animals were anesthetized with IM injections of ketamine (90 mg/kg) and xylazine (10 mg/kg). Livers were excised and sera collected as described in Experiment I.

2.5. Analytical methods

2.5.1. Determination of UCB content

Cells and tissues were sonicated on ice, extracted with methanol/chloroform/hexane 5/5/1 (v/v/v) against PBS buffer pH 6.2 and the organic phase was subsequently extracted into 100 μL of carbonate buffer pH 10. The resulting polar droplet was loaded onto C-8 reverse phase column (Phenomenex, Torrance, CA, USA) and UCB was determined using HPLC with diode array detector (Agilent, Santa Clara, CA, USA) as described earlier [17]. The concentration of UCB was calculated as nmol/g of wet tissue or pmol/mg of protein for tissue samples or cell cultures, respectively.

2.5.2. Determination of lipid peroxidation

Brain tissue from Wistar rats was sonicated and mixed with defined amounts of UCB/BSA. Samples of HepG2 cells were sonicated and diluted to equal protein concentration. Samples were incubated for 30 min at 37 °C with 100 μM ascorbate and 6 μM $FeSO_4$ as described previously [21]. The amount of carbon monoxide (CO) produced into vial was quantitated by gas chromatography with reduction gas detector (GC-RGD) and served as an index of lipid peroxidation.

2.5.3. Determination of HO activity

Cells and tissues were sonicated in PBS. Samples were incubated for 15 min at 37 °C in CO-free septum-sealed amber vials containing MHA (50 μM) and NADPH (4.5 mM). HO activity was determined through measurements of CO production by GC-RGD as described before [20]. HO activity was calculated as pmol CO/h/mg of wet tissue weight or nmol CO/h/mg of protein for tissue samples and cell cultures, respectively.

2.5.4. Determination of BVR activity

Cells were suspended and sonicated in 100 mM Tris buffer pH 8.5 and then incubated with 5 μM biliverdin and 100 μM NADPH for 10 min at 37 °C according to [22]. UCB production was determined by HPLC as described in 2.5.1. The BVR activity was calculated as nmol UCB/h/mg protein.

2.5.5. Determination of heme content

Heme content in cell cultures was determined using HPLC with diode array detector (Agilent, Santa Clara, CA, USA). Samples in 100 μL of deionized water with 1 nmol of internal standard protoporphyrin IXα were sonicated on ice. Then 10 μL of 10 M NaOH and 100 μL of acetone were added. The samples were vortexed and centrifuged at 5000× g for 5 min. The resulting supernatants were loaded onto phenyl-hexyl HPLC column (Phenomenex) equilibrated with methanol/water/tetrabutylammonium phosphate 60/40/0.4 (w/w/w). Analytes were eluted within 18 min using a gradient of 10–80% (v/v) ethanol in the equilibration solvent. The absorbance was measured at 400 nm. The concentration of heme was calculated as ng/mg of protein.

2.5.6. Determination of intracellular free radicals

Intracellular levels of free radicals were assessed using 5-(and-6)-chloromethyl-2′7′-dichlorodihydrofluorescein diacetate acetyl ester (DHF; Invitrogen, Carlsbad, CA, USA) as described elsewhere [23]. The cells were harvested, washed in PBS, and then resuspended in PBS containing 10 μM DHF. After 30 min of incubation at 37 °C, the cells were washed 3 times with PBS and the fluorescein emission was followed spectrofluorimetrically (Shimadzu, Duisburg, Germany). The cellular levels of free radicals were calculated as a fluorescence intensity/mg of protein.

2.5.7. Determination of delta-aminolevulinic acid synthase (ALA synthase) activity

Liver samples were homogenized with glass dust immediately after excision from animals, diluted with Tris buffer pH 7.2 containing 100 mM glycine and incubated at 37 °C for 1 h while intensively shaken. ALA content was measured according to [24]. The ALA synthase activity was estimated as nmol ALA/h/mg of liver tissue.

2.5.8. Determination of malondialdehyde (MDA) content

MDA in tissues was determined using reaction with thiobarbituric acid according to [25].

2.5.9. Serum biochemistry

Total bile acids in rat sera were quantitated using enzymatic colorimetric kit based on 3-hydroxysteroid dehydrogenase activity (Trinity Biotech, Bray, Ireland). Other biochemical markers were analyzed on an automatic analyzer (Modular, Roche Diagnostics GmbH, Germany), using standard laboratory assays.

2.6. Calculation of free UCB (Bf) in media

Bf, an unbound fraction of UCB in media, was calculated according to [26] using equation $Bf = B/(K_F^*(A-B))$, where A is concentration of total BSA, B is concentration of total UCB and K_F is a constant set at 10^7 M^{-1}.

2.7. Statistical analyses

The data in column graphs are presented as mean (column) with SD (error bars) from 4 to 8 samples. Normally distributed results were analyzed using Student's *t*-test. Skewed data were analyzed using Mann–Whitney Rank Sum test. Results were considered significant at $p < 0.05$. Statistical analyses were performed using SigmaStat software (version 3.11, Systat software, San Jose, CA, USA). The correlations were assessed using linear or nonlinear regression in MS Excel (Microsoft corp., Redmond, WA, USA).

3. Results

3.1. The role of exogenous UCB levels

To find a relationship between extracellular and intracellular UCB levels, HepG2 cells were incubated in media containing 0.1–40 μM UCB per 40 g/L of BSA (Bf 0.02–7.1 nM) at 37 °C or 4 °C for 3 h and intracellular UCB was determined. Intracellular UCB levels correlated linearly ($r^2 = 0.998$) with the UCB/BSA ratio in the culture media (Fig. 1A; $p < 0.001$) although there was a proportionally lower accumulation at 4 °C. The antioxidant effects of these doses were then evaluated in a separate experiment on brain tissue homogenates. The addition of UCB/BSA in the same concentration range conferred protection against lipid peroxidation in a dose-dependent manner (Fig. 1B; $p < 0.001$).

3.2. The role of HO-1 induction in vitro

The effect of HO-1 induction on intracellular UCB levels was determined in HepG2 cells cultured in media containing 10 μM UCB per 40 g/L of BSA. After 24 h of incubation with 30 μM heme, a substrate and an inducer of HO-1, a 140-fold elevation in intracellular UCB above control levels was found (Fig. 1C; $p < 0.001$). Simultaneously, HO activity in heme-treated cells increased 2.5-fold compared to controls (4.2 ± 0.5 versus 1.7 ± 0.3 nmol CO/h/mg prot.; $p = 0.01$). Importantly, heme treatment decreased lipid peroxidation of cell lysates 20-fold compared to controls (Fig. 1D; $p < 0.001$). To assess the cellular production of UCB under conditions of naturally limited heme availability, cells were treated with 10–40 μM $NaAsO_2$, a cancerostatic agent [27] and a potent HO-1 inducer [28]. Interestingly, biphasic dose responses of intracellular UCB and HO activity were observed. The intracellular UCB levels increased up to 2.4-fold (Fig. 2A; $p = 0.01$) in cells treated with 10 μM and 20 μM $NaAsO_2$, but not in cells with 40 μM $NaAsO_2$. The increase in HO activity showed the same pattern (Fig. 2B; $p < 0.001$), while BVR activity clearly dropped (Fig. 2D; $p = 0.05$). Surprisingly, total cellular heme was not depleted, but slightly increased in $NaAsO_2$-treated cells (Fig. 2C; $p = 0.03$). The simultaneous inhibition of HO activity with 25 μM SnMP [29] significantly decreased intracellular UCB levels in all treatment groups (Fig. 2A; $p = 0.01$).

3.3. The effect of oxidative stress and HO-1 induction in vitro

In subsequent experiments, the role of HO upregulation in maintaining UCB homeostasis during oxidative stress was studied. The first *in vitro* model of oxidative damage was based on the treatment of HepG2 cells with 100 μM sodium azide [30]. Induction of mild oxidative stress was confirmed by a 1.4-fold increased fluorescence of oxidized DHF (data not shown; $p = 0.002$). Addition of sodium azide to cells cultured in media containing 10 μM UCB per 40 g/L of BSA led to a 60% drop in intracellular UCB compared to controls (Fig. 3A; $p < 0.001$), whereas the simultaneous HO-1 upregulation with $NaAsO_2$ (20 μM) resulted in the complete normalization of intracellular UCB content (Fig. 3A). In addition, sodium azide treatment of cells cultured in media with 4-fold higher UCB concentration (40 μM) resulted in only a 30% decrease of intracellular UCB levels compared to controls (Fig. 3B; $p < 0.001$). Importantly, sodium azide treatment did not affect HO or BVR activity (data not shown).

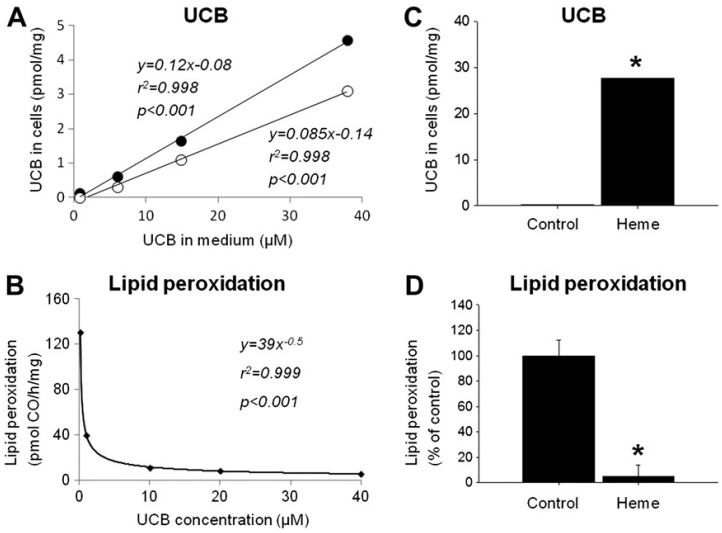

Fig. 1. Relationship between UCB levels and the susceptibility to lipid peroxidation. (A) HepG2 cells were cultured in media with UCB concentrations 0.1–40 μM per 40 g/L of BSA for 3 h at 37 °C (●) or 4 °C (○), and UCB levels were determined in cells and media. (B) Rat brain tissue was treated with increasing doses of UCB (0.1–40 μM per 40 g/L of BSA) and the susceptibility to lipid peroxidation was determined. Data were obtained from 4 independent experiments and analyzed using linear and nonlinear regression, respectively. (C) HepG2 cells were treated with 30 μM heme for 24 h. Intracellular UCB levels and (D) the susceptibility to lipid peroxidation were determined. The data are expressed as mean (column) with SD (error bar) from 5 independent experiments. (*) significantly changed compared to control.

The second *in vitro* model of oxidative damage involved the treatment of cells with TCA [10]. In agreement with the results above, treatment with TCA alone caused a 50% reduction in intracellular UCB (Fig. 3C; $p = 0.05$), while the simultaneous incubation with $NaAsO_2$ (20 μM) returned intracellular UCB to control levels (Fig. 3C). In contrast to sodium azide treatment, TCA caused a significant reduction in cellular HO activity (1.15 ± 0.25 versus 1.67 ± 0.28 nmol CO/h/mg prot.; $p = 0.005$), which was normalized by the simultaneous treatment with $NaAsO_2$ (1.78 ± 0.10 nmol CO/h/mg prot.).

3.4. The effect of HO-1 induction and oxidative stress in vivo

In the first animal study, Wistar rats were treated with LPS to reveal the distribution of bilirubin in serum and tissues under conditions of oxidative stress accompanying a systemic inflammatory insult [31,32]. LPS exposure led to significant elevations of serum markers of hepatocellular and cholestatic liver injury (Table 1), to a 4-fold elevation in total serum bilirubin levels ($p = 0.01$) and a 2-fold elevation in serum UCB (Table 1; $p = 0.002$). Importantly, LPS caused an upregulation of liver HO activity

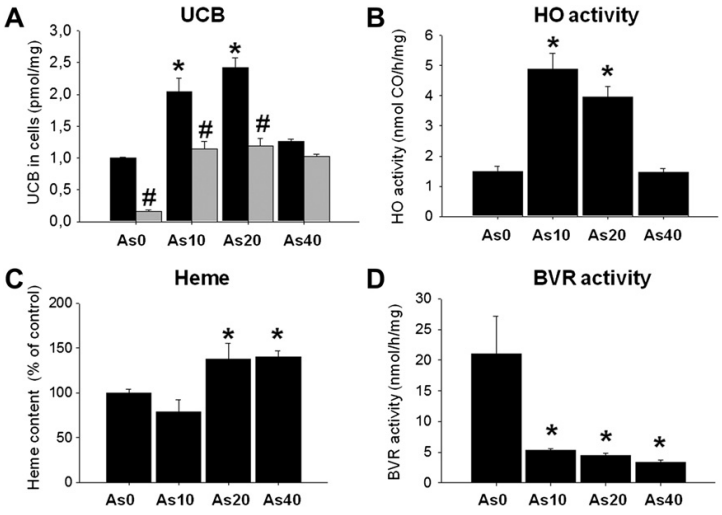

Fig. 2. The effect of sodium arsenite treatment on UCB metabolism. HepG2 cells were treated with $NaAsO_2$ (0, 10, 20 and 40 μM; black bars) or with $NaAsO_2$ and 25 μM SnMP (gray bars) for 24 h. (A) Intracellular UCB levels, (B) HO activity, (C) Intracellular heme levels and (D) BVR activity were determined. The data are expressed as mean (column) with SD (error bar) from 8 independent experiments. (*) significantly changed compared to As0, (#)significantly decreased compared to sample without SnMP.

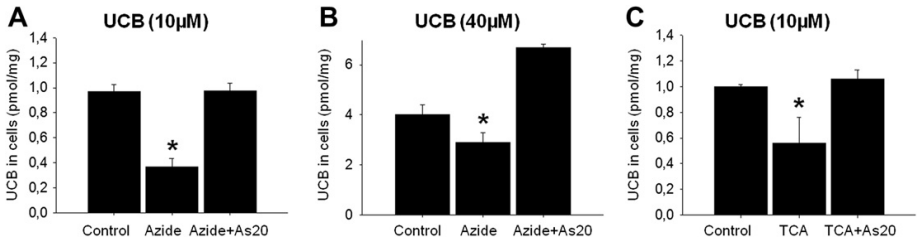

Fig. 3. Intracellular UCB levels in cells under oxidative stress. (A) HepG2 cells cultured in media with 10 μM UCB were treated with either 100 μM sodium azide or with a combination of the former with 20 μM $NaAsO_2$ for 24 h, and intracellular UCB was determined. (B) HepG2 cells cultured in media with 40 μM UCB were treated with 100 μM sodium azide or with a combination of the former with 20 μM $NaAsO_2$ for 24 h and intracellular UCB were determined. (C) HepG2 cells cultured in media with 10 μM UCB were treated with either 50 μM TCA or with a combination of the former with 20 μM $NaAsO_2$ for 24 h, and intracellular UCB was determined. The data are expressed as mean (column) with SD (error bar) from 8 independent experiments. (*) significantly decreased compared to control.

(Table 1; $p = 0.002$) and liver ALA synthase activity (Table 1; $p = 0.02$). These changes were accompanied by a 1.8-fold elevation in liver tissue UCB (Table 2; $p = 0.02$) and more dramatic 3.3-fold elevation in heart UCB (Table 2; $p = 0.01$). On the contrary, tissue MDA levels dropped significantly in the heart (Table 2; $p = 0.004$) but not in the liver (Table 2).

To further confirm the role of HO-1 induction in intracellular UCB regulation, Wistar rats were treated with heme in a separate study. Compared to the LPS treatment, heme treatment resulted in a stronger increase in liver HO activity (Fig. 4A; $p < 0.001$) accompanied with an 8-fold elevation in liver UCB (Fig. 4B; $p < 0.001$). In contrast, the elevation of total serum bilirubin levels was only 2-fold (Fig. 4C; $p = 0.005$).

4. Discussion

The present study was designed to characterize the metabolism of UCB in cells and tissues under oxidative stress, and to identify mechanisms responsible for the regulation of intracellular UCB levels.

We found a correlation between intra- and extracellular UCB levels in HepG2 cells and also elevated tissue UCB levels in animals with cholestasis-associated hyperbilirubinemia. These results demonstrate that under basal conditions, intracellular UCB is in equilibrium with extracellular UCB levels. However, the lower accumulation of intracellular UCB at 4 °C suggests the presence of active import mechanism [33]. In addition, the higher content of UCB significantly decreases tissue lipid peroxidation. These results may account for the protective effects of unconjugated hyperbilirubinemia described in Gilbert's subjects [2] and newborns with neonatal jaundice [34,35]. Therefore, and in agreement with other studies [7,8], we hypothesize that downregulation of liver bilirubin glucuronosyl transferase (UGT1A1) [36] and canalicular exporter MRP2 [37] during cholestasis-associated hyperbilirubinemia represent a systemic protective response to oxidative insult, instead of being a simple consequence of liver dysfunction.

In addition, we found that heme-mediated HO-1 induction in HepG2 cells led to an even more pronounced elevation of intracellular UCB, which was associated with a dramatic decrease in lipid peroxidation. In accord, heme-treated rats showed significant accumulation of UCB in the liver tissue and serum. This is in agreement with our previous study [17] showing that tissue UCB levels in spleen, which is the principle site of hemoglobin breakdown, are 1–2 orders of magnitude higher compared to other tissues. Therefore, we hypothesize that the increased production and retention of UCB could be, at least partially, responsible for the recently reported protective effects of heme supplementation on various syndromes including cadmium-induced testicular damage [38], dextran sulfate-induced colitis [39], and even type 2 diabetes [40].

However, the significance of intracellular UCB elevation following HO-1 upregulation by various non-substrate inducers has been questioned due to the limitation by cellular heme availability [41,42]. Our data show for the first time that intracellular UCB levels in HepG2 cells are significantly elevated after HO-1 induction with low doses of $NaAsO_2$ (10–20 μM), an HO-1 inducer and oxidative stressor [28]. The elevation is abolished upon HO inhibition with SnMP [29] but seems unaffected by the observed drop in BVR activity, probably due to redundant capacity of intracellular BVR. Surprisingly, total cellular heme is unchanged or rather increased in these cells suggesting that the HO-1 induction might be accompanied with an upregulation of the heme biosynthetic pathway. These observations are further supported by the results from LPS-treated rats demonstrating an upregulation of liver HO-1 and liver ALA synthase, a key enzyme in the heme biosynthetic pathway, accompanied by an elevation of liver and serum UCB. This is in agreement with previous reports showing an upregulation of ALA synthase upon HO-1 induction in liver of acetaminophen-treated rats [24]. Moreover, since HO-1 is translocated into

Table 1
Serum markers of hepatocellular/cholestatic liver injury and liver enzyme activities in LPS-exposed Wistar rats.

Marker	Controls	LPS
AST (μkat/L)	1.9 ± 0.3	6.7 ± 3.5*
ALT (μkat/L)	1.2 ± 0.3	5.7 ± 3.1*
ALP (μkat/L)	1.6 ± 0.1	3.7 ± 1.0*
BA (μM)	<8	64 ± 5*
TBIL (μM)	2.4 ± 0.5	9.3 ± 5.0*
UCB (μM)	0.86 ± 0.08	1.8 ± 0.7*
ALAS	1.00 ± 0.07	2.95 ± 0.87*
HO	1.00 ± 0.08	1.47 ± 0.17*

ALAS, delta-aminolevulinic acid synthase (activity relative to control); AST, aspartate amino transferase; ALT, alanine amino transferase; ALP, alkaline phosphatase; BA, total bile acids; HO, heme oxygenase (activity relative to control); TBIL, total bilirubin; UCB, unconjugated bilirubin; (*) significantly increased compared to controls.

Table 2
Different levels of tissue UCB and MDA in LPS-exposed Wistar rats.

Marker	Tissue	Controls	LPS
UCB	Liver	0.9 ± 0.2	1.6 ± 0.6*
	Heart	0.12 ± 0.03	0.4 ± 0.3*
MDA	Liver	1.00 ± 0.16	0.85 ± 0.16
	Heart	1.00 ± 0.17	0.57 ± 0.17*

MDA, malondialdehyde; UCB, unconjugated bilirubin; (*) significantly changed compared to controls.

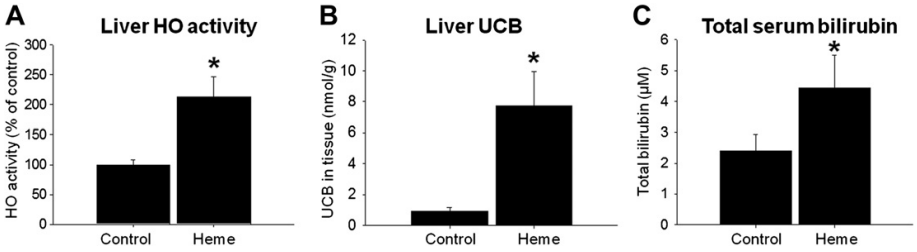

Fig. 4. Metabolism of bilirubin in heme-treated animals. Wistar rats were treated with either saline or heme (2× 10 mg/kg i.p.) for 5 days. (A) Liver HO activity, (B) liver UCB levels, and (C) total serum bilirubin were determined. The data are expressed as mean (column) with SD (error bar) from 8 independent animals. (*) significantly increased compared to control.

mitochondria upon stress induction [42], and final steps of heme synthetic pathway are also localized to mitochondria, we hypothesize that heme synthesis and breakdown are coupled into a cycle, which produces UCB without affecting intracellular free iron levels. This hypothesis agrees with the finding that HO-1 induction with $NaAsO_2$ in HepG2 cells does not necessarily lead to alterations in iron metabolism [41].

The rate-limiting role of HO-1 activity for intracellular UCB accumulation is further supported by our previous studies showing that HO-1 induction with statins caused UCB elevation in myocardial tissue [18], while a decline of HO-1 associated with obstructive cholestasis leads to a significant drop of UCB in the liver [10]. Therefore, our results suggest that the protective effects of HO-1 induction may be at least to some extent mediated through intracellular accumulation of UCB.

More importantly, the protective effects of UCB, heme and $NaAsO_2$ shown in this study are relevant only at relatively low doses of these compounds, while higher doses could show pro-oxidant and toxic effects. Therefore, we employed cell culture media with UCB/BSA ratios adjusted to the range, where Bf (the bioavailable fraction of serum UCB) was more than order of magnitude below the toxic threshold 70 nM [43]. This was not the case for $NaAsO_2$, where higher doses (40 μM) led to a gradual decrease of HO activity accompanied with a lower accumulation of intracellular UCB. This biphasic dose response of UCB to the concentration of $NaAsO_2$ is in agreement with the concept of "hormesis", which states that low doses of stressors cause beneficial induction of the protective responses without significant damage to the organism, while higher doses result in harmful effects [44]. Therefore, we tested whether the treatment with low doses of $NaAsO_2$ could serve as a mechanism maintaining intracellular UCB levels during oxidative stress.

Indeed, changes of intracellular UCB after treatment with sodium azide or TCA confirmed this hypothesis. Sodium azide, a potent inhibitor of mitochondrial cytochrome c oxidase and certain peroxidases, was chosen to induce excessive production of mitochondrial superoxide and hydrogen peroxide [30] while TCA served as an activator of lipid peroxidation and potent HO-1 suppressor [10]. The treatment of HepG2 cells with these compounds led to a dramatic depletion of intracellular UCB which was significantly attenuated by enhanced extracellular UCB availability under simulated hyperbilirubinemic conditions and even completely ameliorated by increased production of UCB by HO-1. These results are further supported by the data from LPS-treated rats demonstrating that liver tissue exposed to cholestatic damage show relatively lower accumulation of UCB and higher degree of lipid peroxidation compared to heart tissue. Taken together, these results suggest that even though the cell culture medium is well supplemented with UCB, diffusion or transport of UCB into cells is insufficient to completely make-up for its loss during severe oxidative stress and thus *de novo* UCB production via the upregulation of HO-1 is required to maintain cellular antioxidant capacity.

In conclusion, our data demonstrate that intracellular metabolism of UCB reflects various conditions of oxidative stress. Both cellular HO-1 induction and systemic hyperbilirubinemia tend to increase the intracellular concentrations of UCB, thus protecting against lipid peroxidation. The presence of active mechanisms regulating cellular UCB availability confirms the physiological significance of UCB in cytoprotection. Thus, pharmacological induction of HO-1 or elevation of serum UCB might prevent various oxidative stress-related syndromes including atherosclerosis, cancer, and type 2 diabetes.

Conflict of interest statement

The authors confirm that there are no conflicts of interest.

Author contribution

Contribution to the work JZ, LM and LV designed the study; JZ, MZ and KV performed the experiments; HJW and RJW contributed the GC-RGD for the study; JZ, LM, HJV, RJW and LV analyzed the data and wrote the paper. All authors have approved the final article.

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Research paper

Protective effects of inhaled carbon monoxide in endotoxin-induced cholestasis is dependent on its kinetics

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ABSTRACT

Carbon monoxide (CO), a product of heme oxygenase (HMOX), has many beneficial biological functions and is a promising therapeutic agent for many pathological conditions. However, the kinetics of inhaled CO and its protective role in endotoxin-induced cholestasis is not fully known. Thus, our objective was to characterize the kinetics of inhaled CO and then investigate its use in early phase experimental endotoxin-induced cholestasis.

Female Wistar rats were randomly divided into 4 groups: CON (control), LPS (lipopolysaccharide, 6 mg/kg), CO (250 ppm COx1h), and CO + LPS. Rats were sacrificed at 0–12 h after LPS administration. Tissues and blood were collected for liver injury markers and tissue CO distribution measurements. Livers were harvested for measurements of Hmox activity, *Hmox1* mRNA expression, cytokines (*IL10*, *IL6*, *TNF*), and bile lipid and pigment transporters.

Half-lives of CO in spleen, blood, heart, brain, kidney, liver, and lungs were 2.4 ± 1.5, 2.3 ± 0.8, 1.8 ± 1.6, 1.5 ± 1.2, 1.1 ± 1.1, 0.6 ± 0.3, 0.6 ± 0.2 h, respectively. CO treatment increased liver *IL10* mRNA and decreased *TNF* expression 1 h after LPS treatment and prevented the down-regulation of bile acid and bilirubin hepatic transporters (*Slc10a1*, *Abcb11*, and *Abcc2*, $p < 0.05$), an effect closely related to the kinetics. The protective effect of CO against cholestatic liver injury persisted even 12 h after CO exposure, as shown by attenuation of serum cholestatic markers in CO-treated animals.

CO exposure substantially attenuated endotoxin-induced cholestatic liver injury and was directly related to the kinetics of inhaled CO. This data underscores the importance of the kinetics of inhaled CO for the proper design of experimental and clinical studies of using CO as a treatment strategy.

1. Introduction

Carbon monoxide (CO) is a ubiquitous air pollutant and toxic gas, but also is an important endogenous signaling molecule, which regulates many biological functions in the body. This product of the heme catabolic pathway, which is catalyzed by the enzyme heme oxygenase (HMOX), plays an important role in inflammation, cell proliferation and cytoprotection [1,2] and thus has a considerable therapeutic potential. Inhalation of low doses of CO has been shown to have potent cytoprotective properties in animal models of organ injury and disease [3]. According to the National Institutes of Health clinical trial database, the CO inhalation model is currently being used in studies investigating its use in the treatment of lung and cardiac diseases (ClinicalTrials.gov; identifier: NCT00094406, NCT01727167, accessed 12th March 2013). To evaluate the safety and effectiveness of using of CO inhalation as a treatment modality, it is critical to not only closely monitor blood CO-hemoglobin (COHb) levels in order to prevent CO poisoning; but also, to identify the optimal concentration of CO that needs to be delivered to target tissues. However, current knowledge about the kinetics of inhaled CO is still very limited.

Cholestasis is characterized as an impairment of bile formation and/or outflow. Although it is a serious complication of sepsis, the pathogenesis of cholestasis is still not fully known [4]. However, it has been shown that inflammatory cytokines released by

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endotoxins can down-regulate expression of hepatobiliary transporters and subsequently impair transport function and bile formation [4,5].

Anti-inflammatory and pro-inflammatory cytokines have been shown to be regulated by the actions of CO via different mechanisms in both *in vivo* [6,7] and *in vitro* studies [6,7]. Nevertheless, a direct effect of CO on hepatic transporters *in vivo* has not been described. Yet, CO has been shown to be an important factor in maintaining the balance between liver sinusoidal perfusion and biliary transport [8,9]. Furthermore, CO is recognized as a regulator of bile canalicular contractility [10]. Taken together, we believe that endogenous CO appears to play an important role in not only inflammation; but also, in the regulation of bile flow and liver integrity.

Therefore, the objective of this study was to first assess the kinetics of inhaled CO administration and then determine its potential use as a treatment for endotoxin-induced liver injury using a rat model.

2. Methods

2.1. Reagents

Bovine serum albumin (BSA), hemin, reduced nicotinamide adenine dinucleotide phosphate (NADPH), sulfosalicylic acid (SSA), ethylenediaminetetraacetic acid (EDTA), RNAlater, lipopolysaccharide from *Escherichia coli* 0111:B4 (LPS) were all purchased from Sigma–Aldrich (St. Louis, MO, USA). The CO (250 ppm) gas mixture and calibration gas (10 ppm) were purchased from Linde Gas (Prague, Czech Republic).

2.2. Animals and in vivo animal studies

Female Wistar rats (190–250 g), obtained from Anlab (Prague, Czech Republic), were allowed water and standard granulated diet *ad libitum*. All animal studies met the criteria for the care and use of animal experiments, and were approved by the Animal Research Committee of the 1st Faculty of Medicine, Charles University in Prague.

Rats were divided into the 4 experimental groups: (1) control group [CON], which received saline intraperitoneally (IP) in the same volume as endotoxin-treated animals; (2) endotoxin-treated group [LPS], which received 6 mg/kg of LPS in saline IP; (3) CO-treated group [CO] which inhaled 250 ppm of CO for 1 h; and received saline IP; and (4) CO- and LPS-treated group [CO + LPS], which received 6 mg/kg of LPS IP immediately after inhalation of 250 ppm CO. At $t = 0$ h or the time of the LPS/saline injection, animals were anesthetized and sacrificed at 0.5, 1, 2, 4, and 12 h ($n \geq 6$ for each time point and group). An aliquot of 100 μL of blood collected from superior vena cava of each animal was transferred to the tubes containing EDTA for COHb measurements [11], and the remaining blood was collected for serum separation. Organs (liver, heart, lung, kidney, spleen, brain, intestine, and muscles) were then harvested and washed in ice-cold reaction buffer (0.1 M potassium phosphate buffer, pH 7.4). For RNA analysis, 100 mg of each tissue was immediately placed in 1.5-mL microfuge tubes containing RNAlater and stored following the manufacturer's protocol till RNA isolation.

2.3. Serum biochemical markers

Serum biochemical markers [alkaline phosphatase (ALP), aspartate aminotransferase (AST), alanine aminotransferase (ALT) and total bilirubin] were determined by standard assays using an automatic analyzer (Modular analyzer, Roche Diagnostics GmbH, Germany). Total serum bile acids (TBA) levels were determined spectrophotometrically (Perkin Elmer UV/VIS spectrometer Lambda 20) using a Bile Acids kit (Trinity Biotech, Jamestown, NY, USA).

2.4. Hmox activity

Freshly harvested livers were diluted 1:9 with 0.1 M potassium phosphate buffer (pH 7.4), minced, and then sonicated with an ultrasonic cell disruptor (Model XL2000, Misonics, Farmingdale, NY, USA). 20 μL of liver sonicate was incubated for 15 min at 37 °C in CO-free septum-sealed vials containing 20 μL of 150 μM methemalbumin and 20 μL of 4.5 mM NADPH as previously described [12]. The amount of CO generated by the reaction and released into the vial headspace was quantified by gas chromatography (GC) with a reduction gas analyzer (Peak Laboratories LLC, Mountain View, CA, USA). Hmox activity, representing combined activities of Hmox1 and Hmox2, was calculated as pmol CO/h/mg fresh weight (FW).

2.5. Tissue CO

Freshly harvested tissues were diluted with ice-cold potassium phosphate buffer (1:4 w/v) and stored in −80 °C until analysis. 40 μL of freshly sonicated sample was added to CO-free, septum-sealed vials containing 5 μL of 30% (w/v) SSA. The vials were incubated for 30 min on ice. CO released into the vial headspace was quantified by GC as previously described [13]. Tissue CO content was expressed as pmol CO/mg FW.

2.6. COHb determination

CO in 1 μL of blood was measured by GC as described previously [11]. Total hemoglobin (tHb) was measured spectrophotometrically at 540 nm after the addition of 4 μL of whole blood to 2 mL of Drabkin's solution. The COHb was expressed as % tHb.

2.7. Kinetic parameters calculation

All concentration data used for kinetic evaluations were corrected for endogenous CO levels in respective tissues of the control group. C_{max} and T_{max} were determined directly from concentration–time profiles of individual animals, CO elimination half-life ($T_{1/2}$) was estimated by the least squares regression analysis, and area under the curve (AUCt) by the linear trapezoidal rule. Non-compartmental analysis using a validated PK solver add-in (China Pharmaceutical University, Nanjing, China) in Microsoft Excel 2010 was used for all kinetic computations [14].

2.8. Real-time RT-PCR analysis of mRNA

Total liver RNA was isolated using Total RNA Purification Kit (Norgen Biotek Corp, Canada) following manufacturer's instructions. High Capacity RNA-to-cDNA Master Mix (Life Technologies, Czech Republic) was used for generating cDNA. Real-time PCR was performed using the TaqMan® Gene Expression Assay Kit for the inducible *Hmox* isoform (*Hmox1*, Rn00561387_m1), interleukin-10 (*IL10*; Rn00563409_m1), interleukin-6 (*IL6*; Rn01410330_m1), tumor necrosis factor-α (*TNF*; Rn99999017_m1), sodium-dependent taurocholate co-transporter (*Slc10a1* coding for Ntcp, a protein responsible for uptake of recirculating conjugated bile acids at basolateral hepatocyte membrane; Rn00566894_m1), multidrug resistance-related protein 2 (*Abcc2* coding for Mrp2, a protein responsible for secretion of conjugated bilirubin into bile; Rn00563231_m1), multidrug resistance protein 3 (*Abcc3* coding for Mrp3, a protein responsible for transport of accumulated bile acids

from hepatocytes into vascular compartment; Rn01452854_m1), sodium-independent organic anion transporting protein (*Slco1a1* coding for Oatp1a1, a basolateral transporter of unconjugated bilirubin and bile acids; Rn00755148_m1), and bile salt export pump (*Abcb11* coding for Bsep, canalicular transporter of bile acids into bile; Rn00582179_m1) genes (Life Technologies, Czech Republic). Data were normalized to β-2 microglobulin (Rn00560865_m1) and expressed as fold change from control levels.

2.9. Statistics

All data are expressed as mean ± SD. For normally-distributed datasets, Student *t*-test and one-way ANOVA with post-hoc Holm–Sidak test for multiple comparisons were used for analysis. For non-normally-distributed data sets, Mann–Whitney rank sum test and nonparametric Kruskal–Wallis ANOVA with Dunn's correction were used. *p*-Values less than 0.05 were considered statistically significant.

3. Results

3.1. Kinetic profile of gaseous CO in vivo

COHb levels were 12.8 ± 1.9% of total hemoglobin (or 2116 ± 152 pmol CO/mg FW), a concentration considered to be safe in rats [15].

The kinetic profiles of inhaled CO were biphasic (fast and slow phases) in all the tissues. The rate of distribution to all the tissues occurred with no apparent delay. We noted non-zero T_{max} values ($n \leq 2$) for liver, spleen, and heart, while there were no non-zero T_{max} samples in all the other tissues. CO levels in all selected tissues returned to control concentrations within 4–12 h after inhalation. No measurable amounts of inhaled CO were found in skeletal muscle and intestine. CO content in the spleen, lungs, heart, liver, kidney, and brain CO were 3.5, 11, 23, 23, 28, and 119 times lower than that of blood (Table 1) after CO exposure. Maximum CO levels were significantly elevated over CON animals as follows: blood (111-fold), spleen (32-fold), lungs (30-fold), heart (9-fold), liver (10-fold), kidney (8-fold), and brain (4-fold) (Table 2).

Mean half-life values ranged from 0.6 h in the lungs and liver to 2.3 h in blood (Table 1) corresponding to apparent tissue-specific elimination rate constants from 1.4 to 0.4 L/h, respectively. Mean residence times ranged from 0.6 h in lung tissue to 1.2 h in blood. The largest interindividual variability of the terminal elimination phase was observed for brain tissue with c_v of approximately 80% for all the computed kinetic parameters as compared to approximate c_v of 35% observed for $T_{1/2}$ and MRT in blood.

The kinetics of inhaled CO was non-linear with more than proportional increases in the rate of elimination with increasing blood concentrations. Administration of LPS did not significantly affect CO elimination (data not shown).

Table 1
Kinetics of inhaled CO in measured tissues.

Tissue	AUC_{0-t} (pmol/mg h)	AUC_{0-2} (pmol/mg h)	$T_{1/2}$ (h)	MRT (h)	λ_z (1/h)
Blood	1783 ± 385	1525 ± 3310	2.3 ± 0.8	1.2 ± 0.4	0.4 ± 0.2
Spleen	511 ± 170	404 ± 145	2.4 ± 1.5	2.5 ± 1.1	0.4 ± 0.2
Lungs	161 ± 52	153 ± 43	0.6 ± 0.2	0.6 ± 0.2	1.4 ± 0.2
Heart	78 ± 38	71 ± 32	1.8 ± 1.6	1.4 ± 1.0	1.2 ± 0.4
Liver	77 ± 20	67 ± 14	0.6 ± 0.3	1.0 ± 0.3	1.4 ± 0.7
Kidney	64 ± 17	60 ± 15	1.1 ± 1.1	1.0 ± 0.6	1.0 ± 0.5
Brain	15 ± 10	8 ± 4	1.5 ± 1.2	2.1 ± 1.7	1.4 ± 1.1

AUC = area under the curve (AUC_{0-t} up to the last measurable concentration, AUC_{0-2} for the first 2 h from end of inhalation), $T_{1/2}$ = CO elimination half-life, MRT = mean residence time, λ_z = terminal elimination rate constant.

Table 2
Uncorrected maximum of CO concentrations in tissues after its inhalation.

Tissue	Control (pmol CO/mg FW)	CO (pmol CO/mg FW)	Fold change
Blood	19 ± 6	2116 ± 152*	111.4
Spleen	14 ± 1	443 ± 45*	31.6
Lungs	8 ± 1	241 ± 42*	30.1
Heart	11 ± 3	102 ± 27*	9.3
Liver	7 ± 1	71 ± 10*	10
Kidney	12 ± 1	92 ± 15*	7.7
Brain	3 ± 1	13 ± 2*	4.3
Muscle-thigh	7 ± 1	9 ± 4	1.3
Muscle-ribcage	8 ± 2	8 ± 0	1
Intestine	5 ± 1	10 ± 7	2

Maximum CO concentrations in tissues (expressed as pmol CO/mg fresh weight) were measured after termination of 1 h inhalation of 250 ppm CO [CO] and compared to levels of animal kept 1 h in room air [control]. The fold change was calculated as the change after inhalation to basal measured levels of CO tissue. *$p < 0.05$ vs. control.

3.2. LPS increases liver Hmox1 mRNA levels and Hmox enzyme activity

To detect if the expression and activity of the stress-inducible and organ-protective enzyme Hmox, the major source of endogenous CO, might be affected by CO treatment, mRNA levels of *Hmox1* and Hmox enzyme activity were measured in the liver. The

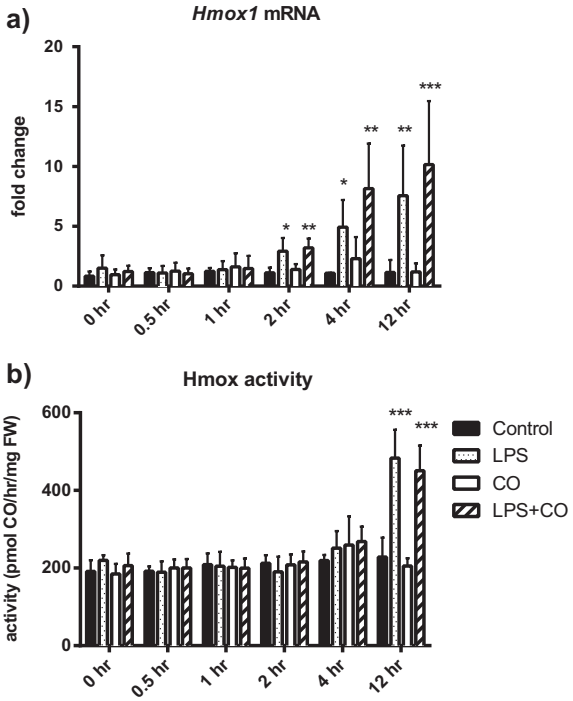

Fig. 1. The effect of LPS and CO on a) *Hmox1* mRNA expression and b) Hmox activity in the liver. a) *Hmox1* mRNA was significantly up-regulated 2 h after LPS administration in LPS- and LPS + CO-treated groups and gradually increased over time. b) Hmox activity significantly increased after 12 h in both LPS- and LPS–CO-treated groups. *$p < 0.05$ vs. control, **$p < 0.01$ vs. control, ***$p < 0.0001$ vs. control.

expression of *Hmox1* mRNA gradually increased starting at 2 h in rats exposed to LPS (LPS only and CO + LPS). Hmox activity significantly increased 12 h after treatment with LPS or CO + LPS (483 ± 73 and 451 ± 65 pmol CO/h/mg FW, respectively, compared to control levels of 228 ± 50 pmol CO/h/mg FW, $p < 0.0001$). Inhaled CO alone did not significantly affect *Hmox-1* mRNA levels or Hmox activity (Fig. 1).

3.3. CO treatment attenuates liver injury

CO treatment prior to LPS administration (CO + LPS group) significantly decreased serum cholestatic markers (ALP and TBA) 12 h after LPS as compared to LPS treatment alone (1.7 ± 0.5 vs. 2.7 ± 0.5 μkat/L and 18.1 ± 13.6 vs. 48.3 ± 30.7 μmol/L, $p < 0.02$, respectively). In addition, AST activities, a marker of hepatocellular liver injury, significantly decreased in the CO + LPS-treated rats 12 h after CO inhalation compared to those treated with LPS only (2.4 ± 0.6 vs. 3.4 ± 1.0 μkat/L, $p = 0.04$, respectively). ALT activities were significantly elevated in both LPS-treated groups with a slight, but non-significant decrease at 12 h in CO + LPS group (1.8 ± 1.3 vs. 1.5 ± 0.7 μkat/L, $p = 0.9$). Total bilirubin levels were elevated at 4 and 12 h after LPS injection regardless of CO exposure (Fig. 2).

3.4. CO affects inflammatory cytokines expression in the liver

To evaluate the effect of CO and LPS on the early inflammatory state of the liver, we directly measured mRNA levels of selected cytokines in liver tissue (Fig. 3). mRNA levels of anti-inflammatory cytokine *IL10* significantly increased within 30 min of LPS administration; and co-treatment with CO further increased *IL10* expression at all time-points ($p < 0.05$). *TNF* mRNA levels peaked 30 min after LPS treatment; the CO co-treatment resulted in a significant amelioration of *TNF* mRNA within 1 h. Expression of the other pro-inflammatory cytokine *IL6* significantly increased after 1 h of LPS treatment and peaked at 2 h. CO co-treatment did not affect *IL6* expression.

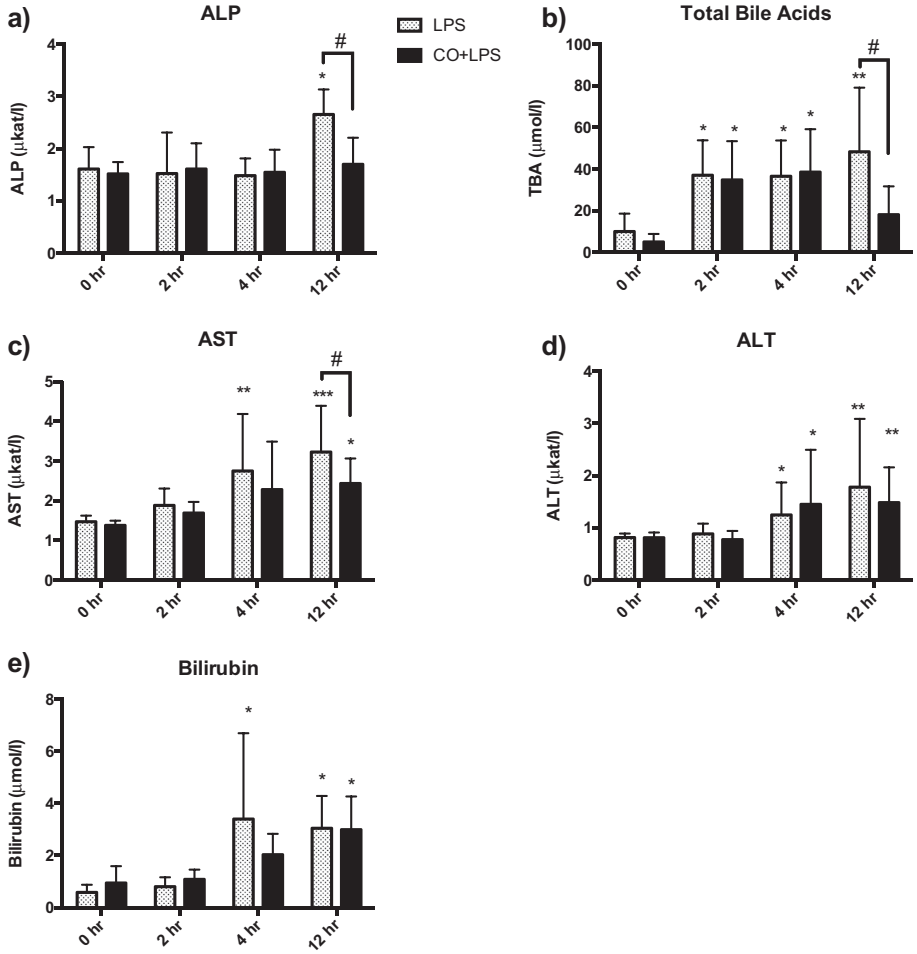

Fig. 2. The effect of LPS and CO exposure on markers of liver injury. Total serum bile acids (TBA), AST, ALT were elevated 4 h after LPS administration. CO pre-treatment decreased the elevation of: a) ALP; b) TBA; and c) AST 12 h after LPS administration. d) ALT activities were significantly elevated from 4 h after LPS injection with no significant changes in the CO + LPS group. e) Bilirubin levels were significantly elevated from 4 h after LPS injection with no significant changes in the CO + LPS group. Control and CO groups did not show any changes for all markers during the experiments (data not shown). $^{*}p < 0.05$ vs. control, $^{**}p < 0.01$ vs. control, $^{***}p < 0.001$ vs. control, $^{\#}p < 0.05$ vs. LPS-treated group.

3.5. CO prevents endotoxin/cholestasis-induced decreases in hepatic transporters expression

We then investigated the effects of CO and LPS on key hepatic transporters within first 12 h of LPS treatment (Fig. 4). At 1 h after LPS-only treatment, we observed a decrease in mRNA of *Abcb11*, *Abcc2* and *Slc10a1* (59 ± 23%, 54 ± 14%, and 49 ± 16%, $p < 0.05$, respectively) from CON levels. Interestingly, these decreases were prevented by CO treatment (CO + LPS group, 92 ± 20%, 92 ± 39%, and 80 ± 36%, $p < 0.05$, respectively). *Abcc2*, *Slc10a1* and *Slco1a1* expressions were significantly attenuated within 12 h in both LPS- and CO + LPS-treated groups; whereas, *Abcb11* and *Abcc3* expressions were not significantly affected by either CO or LPS treatment at this time point.

4. Discussion

In this study, we first characterized in detail the kinetic profile of inhaled CO, and then we correlated these findings to observed changes in expressions of hepatic inflammatory and transporter genes. Initial studies investigating CO kinetics date back to the beginning of 20th century when CO was considered only a waste and toxic product from industrial processes. More than 20 years ago, the beneficial effects of CO were identified and since, CO has been intensively studied for its biological properties [6,9]. As a result of this observation, many scientists have focused on using this powerful molecule as a therapeutic agent. To date, for kinetic model development, only COHb concentrations in blood have been measured and evaluated. While a porcine model showed the CO elimination half-life to be 60.5 min in blood [16], the half-life in an ovine model was shown to be 109–140 min [17]. In our study, we found that the half-life of blood CO in the adult rat after inhalation was 137 ± 47 min, which differs from that reported from studies using COHb measurements. Although species-specific CO kinetics is likely to occur, no data on CO elimination in rats have been available for the comparison.

COHb can be affected by various factors, such as concentration, duration of exposure, time for transport, oxygen saturation and transport, ventilation rate, and the animal model used [18]. Thus, COHb does not seem to be a suitable measure for CO pharmacological profiling. More accurate kinetic modeling, including the blood-to-tissue coefficient have been performed [18], but they have never been confirmed *in vivo*. In 2005, Vreman et al. reported tissue CO levels in mice after CO exposure and found a tissue specificity for CO [13]. In addition to CO distribution, we established detailed elimination profiles for different organs and kinetic parameters for the rat. We found that both CO concentrations and elimination half-lives were tissue-dependent. This observation should be taken into account toward designing studies on the frequency, duration, and the route of CO administration.

Even though CO binding to myoglobin was expected, we observed no increases in CO in skeletal muscle tissues. Vreman et al. showed a 40% increase in CO muscle content after 500 ppm CO inhalation in the mouse [13]. In a subsequent study, they also found high levels of CO in muscles of humans who died from CO inhalation [19]. Sokal et al. [20] showed that the saturation of Mb with CO (COMb) depends on blood COHb levels and not on the duration of exposure to CO. We measured the peak of COHb to be 12.9%, which is less than half of that in the Vreman study (28% of Hb) [13]. The accidental CO exposures in the human study were at the lethal dose [19]. Our observed difference then may be due to the higher dose of CO given in these studies, which in turn may reflect the saturation of binding to Hb first, and then a spilling over to Mb.

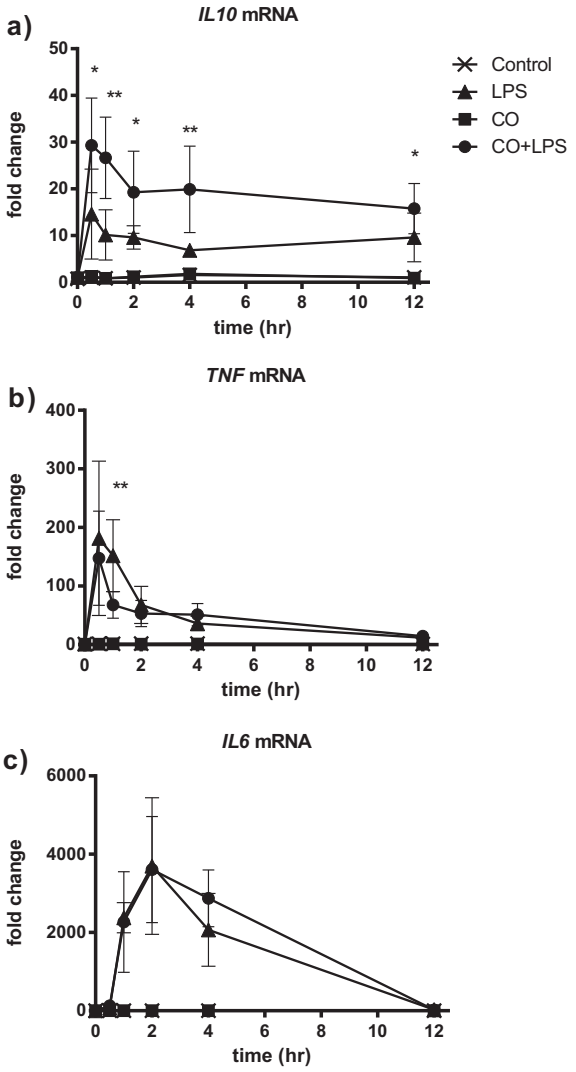

Fig. 3. The effect of CO on expression of inflammatory cytokines in the liver. The expression of cytokines: a) *IL10*; b) *TNF*; and c) *IL6* were measured in hepatic tissue over time. a) Anti-inflammatory *IL10* mRNA was elevated within 0.5 h from LPS treatment at all time points, and significantly increased in CO ± LPS group as compared to the LPS group. b) *TNF* was elevated at 0.5 h and significantly decreased by CO in 1 h after LPS treatment. c) *IL6* was up-regulated in both LPS- and CO + LPS-treated groups. $^{*}p < 0.05$ vs. LPS, $^{**}p < 0.01$ vs. LPS.

The liver plays a key role in the endotoxin metabolism. The development of cholestasis in sepsis and a potential impairment of liver function is a serious complication of sepsis. We have previously shown that bilirubin confers antioxidant protection of hepatic cells in a rat model of obstructive cholestasis [21] suggesting a connection between heme catabolic products and the pathogenesis of cholestasis. Since there is evidence for CO regulating bile flow and liver integrity, we investigated whether exogenous administration of CO might contribute to the resolution of cholestatic liver injury. We found that CO pre-treatment before LPS administration significantly decreased serum cholestatic markers

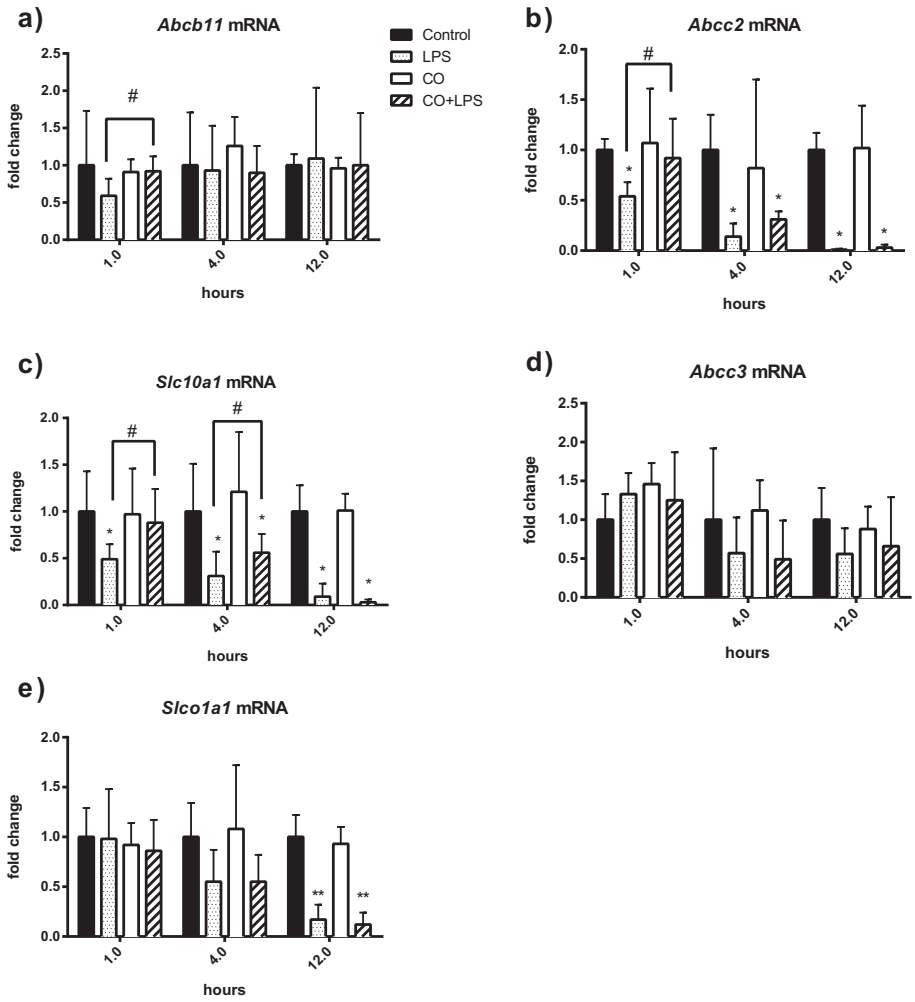

Fig. 4. Effect of CO treatment on expression of hepatic transporters. The expression of hepatic transporter mRNA was measured at different time points after LPS administration. a) *Abcb11* down-regulation was prevented by CO treatment 1 h after LPS; b) *Abcc2* down-regulation induced by LPS was suppressed by CO 1 h after LPS treatment; c) *Slc10a1* expression decrease was suppressed within 1 and 4 h from treatment, but the expression was significantly decreased by LPS in time. d) There are no significant changes in *Abcc3* expression, yet there is a slight increase in 1 h in all treated groups with the later decrease in LPS-treated groups. e) *Slco1a1* expression was slightly decreased by LPS 4 h from the treatment with significant drop in 12 h. $^{*}p < 0.05$ vs. control, $^{**}p < 0.01$ vs. control, $\#p < 0.05$ vs. LPS.

(ALP, bile acids), as well as markers of hepatocellular injury (AST). Interestingly, no effect of CO inhalation on serum bilirubin levels has been observed. We assume that in this case, bilirubin cannot be considered a proper cholestatic marker. In cholestasis, the impaired elimination of bilirubin is responsible for its increased serum levels. In our study, also other effects including the increased synthesis (represented by increased Hmox activity) might substantially influence the final serum bilirubin concentration. In 1994, Luster et al. [22] showed that the administration of LPS increases liver TNFα, IL-6, and IL-1β levels in a rodent model. Later, the inflammatory cytokines were suggested as potent inducers for sepsis-induced cholestasis [4]. We therefore measured the expression of selected cytokines in the liver to investigate whether short-term exposure to low doses of CO can directly modulate inflammatory parameters. We found that the expression of an anti-inflammatory cytokine *IL10* was up-regulated in LPS-treated groups, and even enhanced by CO pre-treatment in the liver. This is in agreement with other studies that demonstrated an anti-inflammatory potential of CO in macrophages and serum [6]. Simultaneously, *TNF* mRNA was significantly down-regulated 1 h after CO treatment, although expression of *IL6* was not affected. It has been shown that administration of IL-10 together with endotoxin decreases the level of TNFα within 2 h and subsequently, the level of TNFα decreases in both IL-10 treated and untreated groups [23]. Thus, in our experiment, the dramatically higher IL-10 mRNA peak at 0.5 h followed by significantly larger drop in TNFα at 1 h in the LPS + CO group compared to LPS only might be explained by this mechanism.

Since inflammatory cytokines can influence the expression of hepatic transporters [5], we investigated if CO pre-treatment can affect the hepatic transporters expression. Administration of non-

lethal dose of LPS to adult rats was reported to down-regulate *Slc10a1* [24], *Abcc2* [25], *Slco1a1* and *Abcb11* [26] transporters, which was confirmed in our study. Interestingly, these effects were partially prevented by CO pretreatment. The major preventive effect of CO on *Abcb11, Abcc2* and *Slc10a1* was observed within 1 h after LPS administration with a persisting moderate effect of CO pre-treatment lasting for 4 h. These findings are in line with our observation demonstrating that more than half of liver CO concentration was excreted before 1 h from the end of inhalation. Even though this effect was only transient, it was sufficient to significantly decrease serum bile acid levels 12 h after LPS administration. The accumulation of bile acids in sera is a dynamic process reflecting the transport ability of hepatocytes in time, and can be assumed that the significant improvement in bile acid concentration in 12 h is the result of hepatocyte uptake and secretion within previous hours. Contrary to reports showing anti-inflammatory effects of CO (reviewed in Ref. [27]), there are also some studies that did not observe any protective effects *in vivo* [28,29]. This discrepancy could be explained by the lack of CO accumulation in the target tissue further substantiating the need to define the kinetics of inhaled CO.

CO was previously shown to display its effects via many different pathways [6,7]. We showed that there is a down-regulation of hepatic transporters by LPS as early as 1 h after the onset of inflammation (or at least the release of endotoxin) that is partially diminished by CO pre-treatment. These early events can positively affect the integrity and transporter function of hepatocytes, which we observed as a subsequent improvement in serum markers of cholestasis and resolution of liver damage.

There are several limitations of our study. First, we can only hypothesize that the positive effects of CO in the early phase of endotoxin-induced cholestasis can confer later protection and alter the development of cholestasis. Further studies to measure late phase responses to a single dose of CO prior to LPS exposure could clarify this question. Secondly, we cannot exclude that there may be a direct effect of inhaled CO on the translation of liver transporters, their stability, protein folding, and incorporation into membranes, phosphorylation or degradation. To address these issues, it will be necessary to perform complex studies with individual transporter proteins.

In conclusion, we found that the distribution and elimination of CO is tissue-dependent with its half-life independent of the CO concentration. In addition, we showed that CO exposure substantially attenuated endotoxin-induced cholestatic liver injury, an effect directly related to the kinetics of inhaled CO. This data underscores the importance of understanding the kinetics of CO for the proper design of experimental and clinical studies of using inhaled CO as a potential treatment strategy.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

Author contribution

KV, LM and LV designed the study, KV, JS, TP, DC performed the experiments, HJV, RJW contributed GC-RGD for the study, KV, OS, HJV, RJW, LV, LM analyzed the data and wrote the paper. All authors approved the final article.

Acknowledgments

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Protective effect of heme oxygenase induction in ethinylestradiol-induced cholestasis

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Abstract

Estrogen-induced cholestasis is characterized by impaired hepatic uptake and biliary bile acids secretion because of changes in hepatocyte transporter expression. The induction of heme oxygenase-1 (HMOX1), the inducible isozyme in heme catabolism, is mediated *via* the Bach1/Nrf2 pathway, and protects livers from toxic, oxidative and inflammatory insults. However, its role in cholestasis remains unknown. Here, we investigated the effects of HMOX1 induction by heme on ethinylestradiol-induced cholestasis and possible underlying mechanisms. Wistar rats were given ethinylestradiol (5 mg/kg s.c.) for 5 days. HMOX1 was induced by heme (15 μmol/kg i.p.) 24 hrs prior to ethinylestradiol. Serum cholestatic markers, hepatocyte and renal membrane transporter expression, and biliary and urinary bile acids excretion were quantified. Ethinylestradiol significantly increased cholestatic markers ($P \leq 0.01$), decreased biliary bile acid excretion (39%, $P = 0.01$), down-regulated hepatocyte transporters (Ntcp/Oatp1b2/Oatp1a4/Mrp2, $P \leq 0.05$), and up-regulated Mrp3 (348%, $P \leq 0.05$). Heme pre-treatment normalized cholestatic markers, increased biliary bile acid excretion (167%, $P \leq 0.05$) and up-regulated hepatocyte transporter expression. Moreover, heme induced Mrp3 expression in control (319%, $P \leq 0.05$) and ethinylestradiol-treated rats (512%, $P \leq 0.05$). In primary rat hepatocytes, Nrf2 silencing completely abolished heme-induced Mrp3 expression. Additionally, heme significantly increased urinary bile acid clearance *via* up-regulation (Mrp2/Mrp4) or down-regulation (Mrp3) of renal transporters ($P \leq 0.05$). We conclude that HMOX1 induction by heme increases hepatocyte transporter expression, subsequently stimulating bile flow in cholestasis. Also, heme stimulates hepatic Mrp3 expression *via* a Nrf2-dependent mechanism. Bile acids transported by Mrp3 to the plasma are highly cleared into the urine, resulting in normal plasma bile acid levels. Thus, HMOX1 induction may be a potential therapeutic strategy for the treatment of ethinylestradiol-induced cholestasis.

Keywords: 17α- ethinylestradiol • heme • nuclear factor erythroid-2-related factor-2 • bile acids • multidrug resistance-associated protein 3

Introduction

Estrogens are known to cause intrahepatic cholestasis in susceptible women during pregnancy, administration of oral contraceptives or hormone replacement therapy [1]. In fact, it is a rather frequent condition, with a prevalence rate of intrahepatic cholestasis of pregnancy reaching 0.2–1.5% in Europe and USA [2]. Induction of cholestasis by the synthetic estrogen, 17α-ethinylestradiol (EE), has

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been used as an experimental model of human intrahepatic cholestasis [3].

The mechanisms involved in EE-induced cholestasis are multifactorial and include reduction in both bile salt-dependent [4] as well as independent [5] bile flow and the subsequent increase of serum bile acids (BA). Functional analyses revealed diminished sinusoidal uptake and canalicular transport of BA caused by down-regulation of the main membrane transporters – sinusoidal NTCP (Na^+-taurocholate co-transporting polypeptide, *SLC10A1*) and OATPs (organic anion-transporting polypeptides, encoded by *SLCOs*) [3, 6]; and canalicular MRP2 (multidrug resistance-associated protein 2, encoded by *ABCC2*) and BSEP (bile salt export pump, encoded by *ABCB11*) [7, 8]. Estrogens are also implicated in reduced bile salt synthesis [9], increased tight junctions permeability [10], decreased plasma membrane fluidity and redistributed gangliosides within hepatocyte membranes [11, 12].

Heme oxygenase (HMOX) is the rate-limiting enzyme in the heme catabolic pathway. It catalyses the degradation of heme to produce equimolar amounts of carbon monoxide (CO), iron and biliverdin, the latter being rapidly metabolized to bilirubin by biliverdin reductase [13]. There are two structurally related HMOX isozymes, the inducible HMOX1 (OMIM*141250), also called heat-shock protein 32 (HSP32), and the constitutive HMOX2 (OMIM *141251) [14]. The induction of HMOX1 by its substrate, heme, is mediated *via* Bach1/Nrf2 (nuclear factor erythroid-2-related factor-2) pathway [15]. Over the past decade, enhanced HMOX enzymatic activity has emerged as an important mediator of antioxidant, cytoprotective, neurotransmitter and anti-inflammatory actions mediated by the production of its bioactive products, CO and bilirubin [16–18]. Moreover, a number of animal as well as clinical studies emphasize the crucial role of HMOX in the protection against oxidative stress-mediated diseases including atherosclerosis [19], diabetes [20], hypertension [21] and cancer [22].

In the liver, the HMOX1 and HMOX2 isozymes have distinct topographic patterns. HMOX1 is expressed predominantly in Kupffer cells, while the constitutive HMOX2 is abundant in hepatocytes [23]. Suemetsu *et al.* [24] have shown that CO derived from HMOX2 is necessary to maintain liver sinusoids in a relaxed state, and this process is mediated by mechanisms involving soluble guanylate cyclase in hepatic stellate cells. *In vivo*, HMOX1 induction has been shown to protect mice and rats from apoptotic liver damage because of liver graft rejection as well as from ischaemia/reperfusion injury [25, 26]. Furthermore, CO contributes to the maintenance of blood perfusion in the liver and to the excretion of bile [27]. In another study, stress-induced levels of CO (up to concentrations of 4–5 μmol/L) were shown to stimulate bile secretion in a dose-dependent manner, although further administration of higher amounts of CO caused a reduction of bile output by mechanisms appearing to involve hepatocyte membrane transporter Mrp2 [28]. In addition, CO has been shown to limit the contractility of bile canaliculi by suppressing intracellular calcium mobilization [29] and modulate the expression of liver transporters [30, 31]. Also, retention of bilirubin, a potent antioxidant product of heme catabolic pathway, might play an important cytoprotective role in cholestasis as well [32].

The objective of this study was to investigate whether induction of HMOX by heme prevents EE-induced cholestasis in rats and to identify the possible underlying mechanisms.

Material and methods

Chemicals

EE, NADPH, hemin, sulfosalicylic acid (SSA), bilirubin, bovine serum albumin, taurocholic acid, glutathione, glutathione-reductase, 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Reagents

Potassium phosphate buffer, 0.1 M, pH 7.4

Dihydrogen potassium phosphate, 13.61 g, was dissolved in distilled water. The pH was adjusted to 7.4 with KOH (0.1 M). The final volume was brought to 1 l with distilled water [33].

Methemalbumin, 0.15 mM

Hemin, 9.9 mg, was dissolved in 2.5 ml of 0.4 M Na_3PO_4. Distilled water was added to a volume of 8 ml and 100 mg of bovine serum albumin was dissolved. The pH was gradually adjusted to 7.4 by using 1.0 N HCl with vigorous stirring. Distilled water was added to bring the total volume to 10 ml [33]. Heme was always administered in the form of methemalbumin to animals or used for *in vitro* experiments.

Animals

Adult female Wistar rats obtained from Anlab (Prague, Czech Republic) weighing 200–280 g, were provided with water and food *ad libitum*. All aspects of the animal studies met the accepted criteria for the care and experimental use of laboratory animals. All protocols were approved by the Animal Research Committee of the 1st Faculty of Medicine, Charles University in Prague.

Rats were randomly divided into 4 groups: (1) those receiving only vehicle (propanediol) or CON; (2) those administered 5 mg/kg EE daily i.p. for 5 consecutive days (days 1–5) or EE; (3) those receiving 15 μmol/kg heme i.p. on days 0 and 3) or HC; and (4) those co-administered heme and EE at the above-mentioned doses or HE. Each group included at least 8 animals.

Surgical procedures were performed on day 6 between 8 and 10 a.m. Experimental animals were anaesthetized with sodium pentobarbital (50 mg/kg i.p.). Biliary trees were then exposed through midline abdominal incisions. Bile ducts were cannulated and bile was collected for 20 min. (equilibration) and then in two 30-min. intervals (20–50 and 50–80). In addition, all rats were cannulated with polyethylene tubes in the left carotid artery for blood sampling and urinary bladder for urine collection. Urine was collected in three sessions: first one for 20 min. (equilibration) and then in two 30-min. intervals (20–50 and 50–80). For biliary and urinary BA output, the 20–50 collections of bile and urine were used. Body temperature was maintained at 37°C by using a

heated platform. At the end of each experiment, animals were killed by exsanguination, and the livers were removed and weighed.

Markers of cholestasis

Serum biochemical markers [alkaline phosphatase (ALP), alanine aminotransferase (ALT), bilirubin] were determined in an automatic analyser (Modular analyser; Roche Diagnostics GmbH, Mannheim, Germany) by using standard assays. Total serum and biliary BA levels were determined spectrophotometrically by using a Bile Acids kit (Trinity Biotech, Jamestown, NY, USA). BA levels in urine were determined by gas chromatographic/mass spectrophotometric method as previously described [34].

Total HMOX enzyme activity determinations

Twenty microlitres of 10% liver sonicate [2 mg fresh weight (FW)] was incubated for 15 min. at 37°C in CO-free septum-sealed vials containing identical volumes of 150 μM heme and 4.5 mM NADPH as previously described [33]. Blank reaction vials contained potassium phosphate buffer in place of NADPH. Reactions were terminated by adding 5 μl of 30% (w/v) SSA. The amount of CO generated by the reaction and released into the vial headspace was quantified by gas chromatography with a Reduction Gas Analyser (Peak Laboratories, Mountain View, CA, USA). HMOX activity was calculated as pmol CO/h/mg FW.

HMOX-1 mRNA determinations

Total liver RNA was isolated by using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and cDNA was generated by using an iScript reverse transcription kit (Bio-Rad Laboratories, Hercules, CA, USA). Real-time PCR was performed with TaqMan® Gene Expression Assay Kit for following genes: *Ntcp* (*Slc10a1*, Rn00566894_m1), *Oatp1a1* (*Slco1a1*, Rn00755148_m1), *Oatp1a4* (*Slco1a4*, Rn00756233_m1), *Bsep* (*Abcb11*, Rn00582179_m1), *Mrp2* (*Abcc2*, Rn00563231_m1), *Mrp3* (*Abcc3*, Rn01452854_m1), *Mrp4* (*Abcc4,* Rn01465702_m1), *Hmox1* (*Hmox1*, Rn00561387_m1), *Asbt* (*Slc10a2*, Rn00691576_m1), *Osta* (*Ostalpha*, Rn01763289_m1) and *Gapdh* rat endogenous control kit, all provided by Life Technologies (Carlsbad, CA, USA).

Biliary total glutathione determinations

Glutathione was determined in a bile sample collected for 20 min. Bile was mixed with five volumes of SSA (5% w/v in distilled water) and stored at −80°C until analysis. Total glutathione was measured as previously described [35]. Briefly, bile samples were first diluted 500-fold by using a phosphate (100 mM)/EDTA (1 mM) buffer (pH 7.4). Diluted bile samples (50 μl) were transferred to 96-well microplate and mixed with 100 μl of recycling agent (containing 0.30 mM NADPH, 0.225 mM DTNB and 1.6 U/ml glutathione-reductase in an EDTA phosphate buffer). Immediately after recycling agent addition, colour development was recorded at 405 nm for 4 min. by using Tecan Sunrise™ microplate reader equipped with kinetic analysis software (Tecan group Ltd., Mannedorf, Switzerland).

Primary rat hepatocyte culture and transient transfection assay

Primary hepatocytes were isolated from anaesthetized Wistar rats by the two-step collagenase perfusion as previously described [36]. Hepatocytes with cell viability greater than 90% (as assessed by trypan blue staining) were first plated on 35-mm collagen-coated cell culture dishes and maintained at 37°C, 5% CO_2 in William's medium E, supplemented with penicillin/streptomycin, L-glutamine, insulin and 10% foetal bovine serum. On the next day, Nrf2 gene was silenced with siRNA (Sigma-Aldrich) by using Lipofectamine RNAiMAX (Invitrogen) according to the manufacturer's instructions. The knockdown level of Nrf2 gene was verified by qRT-PCR and was always higher than 75%. Cells were treated with vehicle, TCA (10, 50, 100 μM), unconjugated bilirubin (25, 250 μM), EE (10 μM) and/or MHA (30 μM) 24 hrs after transfection.

Statistical analyses

Normally distributed data are presented as means ± SD and analysed by Student's *t*-test and one-way ANOVA with post-hoc Holm–Sidak test for multiple comparisons. Non-normally distributed data sets are expressed as medians (25%–75%) and analysed by Mann–Whitney rank sum test and nonparametric Kruskal–Wallis ANOVA with Dunn's correction. Data with highly skewed distributions were log-transformed. Differences were deemed statistically significant when $P < 0.05$.

Results

Induction of HMOX1 normalizes serum BA in cholestatic rats

As expected, compared with controls, induction of cholestasis with EE resulted in significant increases of total BA, ALP activity as well as total serum bilirubin levels (Table 1). Heme pre-treatment resulted in normalization of total BA concentrations as well as ALP activity in cholestatic animals, while total bilirubin levels remained elevated. Application of heme to control animals had no effect on BA and ALP (cholestatic parameters), but total bilirubin levels significantly increased (most likely as a result of bilirubin formation from heme administered to this experimental group). No significant changes have been observed in the serum ALT activity, a marker of hepatocellular liver injury (data not shown).

Total liver HMOX activity in EE-induced cholestasis

No significant differences in HMOX activity were observed in the livers of CON and EE groups (211 ± 22 *versus* 176 ± 27 pmolCO/h/mg FW, respectively, $P = 0.85$). As expected, heme pre-treatment resulted in an increase in liver HMOX activity in both HC and HE groups (353 ± 166 and 290 ± 52 pmolCO/h/mg FW, respectively, $P \leq 0.05$) as compared with CON and EE groups.

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Table 1 The effect of heme pre-treatment on serum cholestatic markers

Groups	Total serum bile acids (μmol/L)	ALP (μkat/L)	Total serum bilirubin (μmol/L)
Vehicle (CON)	26.0 ± 11.9	2.1 ± 0.8	2.0 ± 0.8
EE-treated (EE)	**54.3 ± 22.2***	**3.6 ± 1.1***	**5.2 ± 1.2***
Vehicle + heme (HC)	29.4 ± 17.4	2.2 ± 0.5	**5.1 ± 2.2***
EE + heme (HE)	**23.3 ± 17.2**†	**2.6 ± 1.3**†	**5.5 ± 1.4***

Bile acid (BA) concentrations, alkaline phosphatase (ALP) activity and total bilirubin levels were measured in sera of control (CON), ethinylestradiol (EE), heme (HC), and heme + EE (HE)-treated animals.
*$P \leq 0.05$ *versus CON*, †$P \leq 0.05$ *versus EE*.

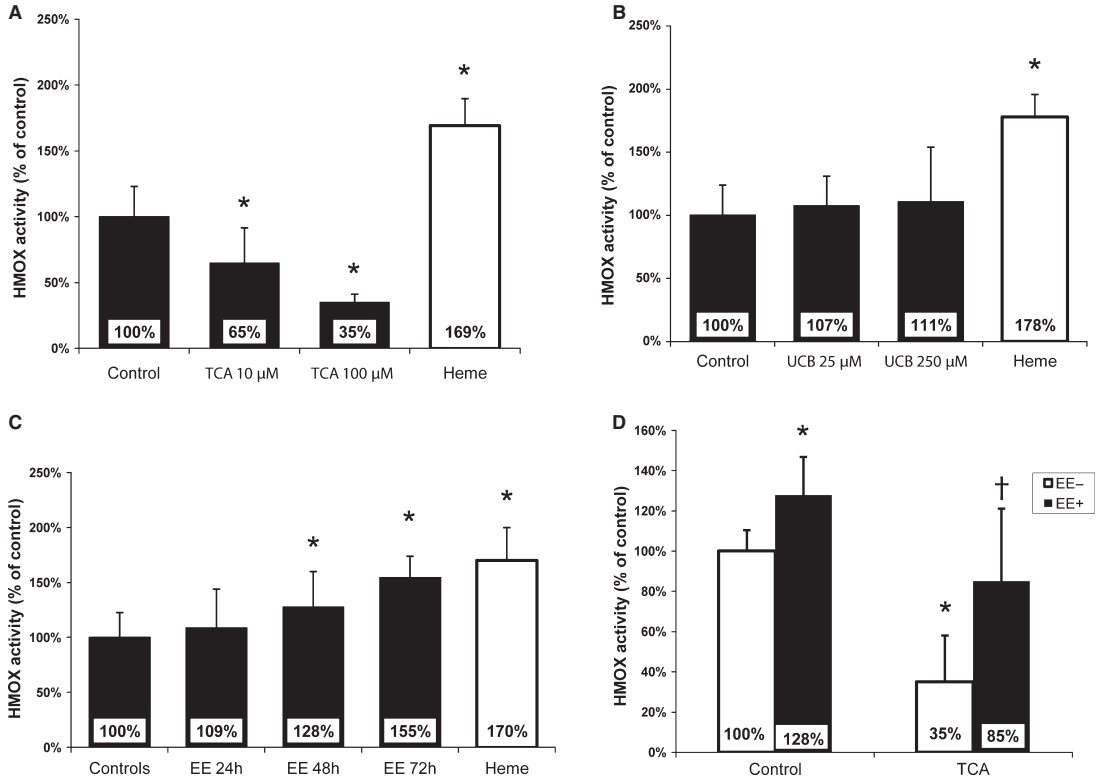

Fig. 1 Total heme oxygenase (HMOX) activity in primary rat hepatocytes. Primary hepatocytes were incubated with: (**A**) taurocholic acid (TCA), (**B**) unconjugated bilirubin (UCB) for 24 hrs or with (**C**) 10 μM ethinylestradiol (EE) for 24, 48 and 72 hrs. (**D**) 10 μM EE was added to media 24 hrs before TCA treatment (100 μM, another 24 hrs). *$P \leq 0.05$ *versus CON*, †$P \leq 0.05$ *versus TCA*. Hepatocytes treated with heme served as positive controls.

Cross-talk between EE and BA in primary rat hepatocytes

Because of the inhibitory effect of BA on HMOX activity in HepG2 cells *in vitro* and in obstructive cholestasis *in vivo* described previously by our group [32], we decided to investigate whether BA, EE, and bilirubin affected total HMOX enzyme activity in primary rat hepatocytes. We found that HMOX activity significantly decreased to 65% and 35% of CON levels 24 hrs after incubation with 10 or 100 μM taurocholic acid, respectively ($P < 0.01$) (Fig. 1A). Bilirubin, ranging from 25 to 250 μM, had no effect on HMOX activity (Fig. 1B). Similarly, incubation with 10 μM EE for 24 hrs had no effect on HMOX activity. However, significant increases were found 48 and 72 hrs (135% and 155%, $P < 0.05$, respectively) after treatment with 10 μM EE

Table 2 Effect of heme oxygenase (HMOX) induction on bile salt-dependent and -independent bile flow

Group of animals	Biliary bile acids output (μmol/g/min.)	Bile flow (μl/g/min.)	Biliary glutathione output (nmol/g/min.)
Vehicle (CON)	210.02 ± 43.59	2.21 ± 0.25	7.43 ± 2.34
EE-treated (EE)	81.64 ± 8.94*	0.54 ± 0.10*	0.32 ± 0.04*
Vehicle + heme (HC)	207.24 ± 12.03	2.13 ± 0.47	9.38 ± 0.17
EE + heme (HE)	135.52 ± 45.07*†	0.81 ± 0.25*†	0.52 ± 0.05*†

Bile volume, bile acids and glutathione concentrations were measured in the bile collected for 30 min. from control (CON), ethinylestradiol (EE), heme (HC) or heme + ethinylestradiol (HE)-treated animals and recalculated to grams of liver tissue.
*$P \leq 0.05$ *versus CON*, †$P \leq 0.05$ *versus EE*.

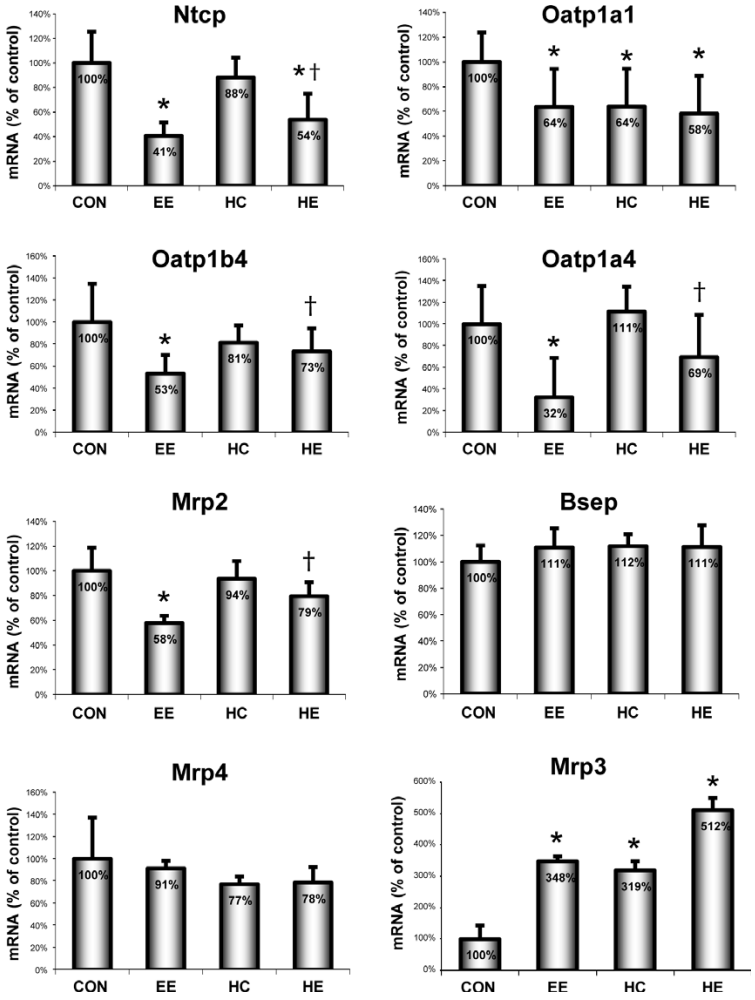

Fig. 2 mRNA expression of key hepatic transporters. Relative expression of key sinusoidal (*Ntcp, Oatps, Mrp3* and *Mrp4)* and canalicular (*Mrp2, Bsep*) bile acid (BA) transporters was measured in the livers of control (CON), ethinylestradiol (EE), heme (HC) or heme + EE (HE)-treated animals. *$P \leq 0.05$ *versus CON*, †$P \leq 0.05$ *versus EE*.

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(Fig. 1C). Moreover, EE treatment significantly diminished the inhibitory effect of taurocholic acid on HMOX activity (Fig. 1D).

Induction of HMOX1 increases biliary secretion of BA and glutathione in cholestatic rats

To identify the role of heme pre-treatment on bile production, we measured bile flow and biliary bile acids secretion rate in control and cholestatic animals with and without heme treatment ($n = 6$ in each group). A significant drop of bile flow to 24% ($P < 0.001$) was observed in EE-treated animals compared with controls. Importantly, heme pre-treatment of cholestatic animals resulted in slight increase of bile flow to 150% ($P = 0.03$) compared with those treated with EE. Administration of heme to control animals had no effect on bile flow (96%, $P = 0.38$).

In addition, biliary BA secretion decreased in cholestatic animals (EE) to 39% of CON values, but significantly increased after heme pre-treatment (HE 166% compared to EE, $P = 0.04$) (Table 2).

To clarify the effect of EE and heme on bile salt-independent bile flow in our experimental settings, we measured the biliary glutathione output in CON and cholestatic rats with or without heme pre-treatment. Compared with CON, biliary glutathione output was significantly reduced in EE-treated animals (to 4%, $P = 0.03$). Administration of heme to EE-treated animals led to an apparent increase (to 162%, $P = 0.006$, *versus* EE) in glutathione output, although the values did not reach CON values. Administration of heme to CON animals had no significant effect on glutathione output (126%, $P = 0.18$) (Table 2).

Effect of heme on expression of hepatocyte transporters

To elucidate the mechanism by which heme stimulates bile flow in EE-treated cholestasis, we measured the expression of key hepatocyte bile pigment and lipid transporters in the rat livers. EE treatment significantly decreased expression of sinusoidal *Ntcp* and *Oatps* (*Oatp1a1*, *Oatp1a4*, *Oatp1b2*) as well as canalicular *Mrp2* transporters. No effect was observed on the expression of sinusoidal *Mrp4* and canalicular *Bsep*. Importantly, heme pre-treatment of EE-exposed rats significantly increased mRNA of key hepatocyte transporters (*Ntcp*, *Oatp 1a4*, *Oatp1b2*, *Mrp2*; Fig. 2). Interestingly, EE as well as heme, up-regulated sinusoidal *Mrp3* expression in CON rats (348% and 319%, respectively, $P < 0.05$). This increase was even more pronounced in cholestatic rats treated with heme (HE) (512%, $P < 0.05$).

In another set of experiments, we focused on mechanism of heme-induced *Mrp3* overexpression. We examined the effect of the main heme-activated transcription factor *Nrf2* on *Mrp3* expression in primary rat hepatocytes (Fig. 3). While treatment of cells with heme or heme + taurocholic acid markedly increased the *Mrp3* expression, the silencing of *Nrf2* led to a significant decrease in *Mrp3* expression in all experimental groups.

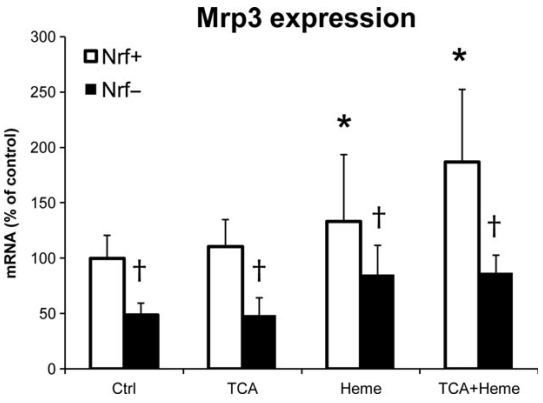

Fig. 3 Mrp3 expression in primary rat hepatocytes. Relative expression of *Mrp3* transporter mRNA was measured in primary hepatocytes with (Nrf−) or without (Nrf+) Nrf2 silencing. Cells were treated with 50 μM taurocholic acid (TCA), 30 μM heme or both for 4 hrs. $*P \leq 0.05$ *versus CON*, $^{\dagger}P \leq 0.05$ *versus corresponding Nrf+ group*.

Induction of HMOX1 increases urinary BA output

The significant increases in *Mrp3* expressions in cholestasis as well as after heme pre-treatment (resulting in an increased transport of conjugated BA from hepatocytes to the bloodstream) together with low serum concentration of BA in HE rats prompted us to measure the extent of urinary BA output. As expected, urinary BA output significantly increased (402%) in cholestatic animals compared with CON ($P = 0.04$). Interestingly, administration of heme to CON animals resulted in a significant increase (217%) in urinary BA output and was even more pronounced in heme-pre-treated cholestatic rats when compared with CON (1183%, $P < 0.05$; Fig. 4A).

Unlike in the liver, the expression of *Mrp3*, a renal BA reabsorption transporter, was significantly decreased following heme administration. On the other hand, heme caused significant increases in the expression of kidney *Mrp2* and *Mrp4*, important renal BA exporters (Fig. 4B). We did not observe any significant changes in the expression levels of *Asbt* and *Osta* (data not shown).

Discussion

In this study, we demonstrated that the induction of HMOX1 with its substrate, heme, can confer protection against EE-induced cholestasis by increasing both liver and renal clearance of BA in a rat model.

Recently, our group has shown that BA down-regulates HMOX activity *in vitro* and *in vivo* [32], and that this effect might be responsible for increased oxidative stress and subsequent liver injury in obstructive cholestasis. However, we did not observe any changes in HMOX activity in the livers of cholestatic EE-treated animals compared with controls. Parallel *in vitro* experiments using primary rat

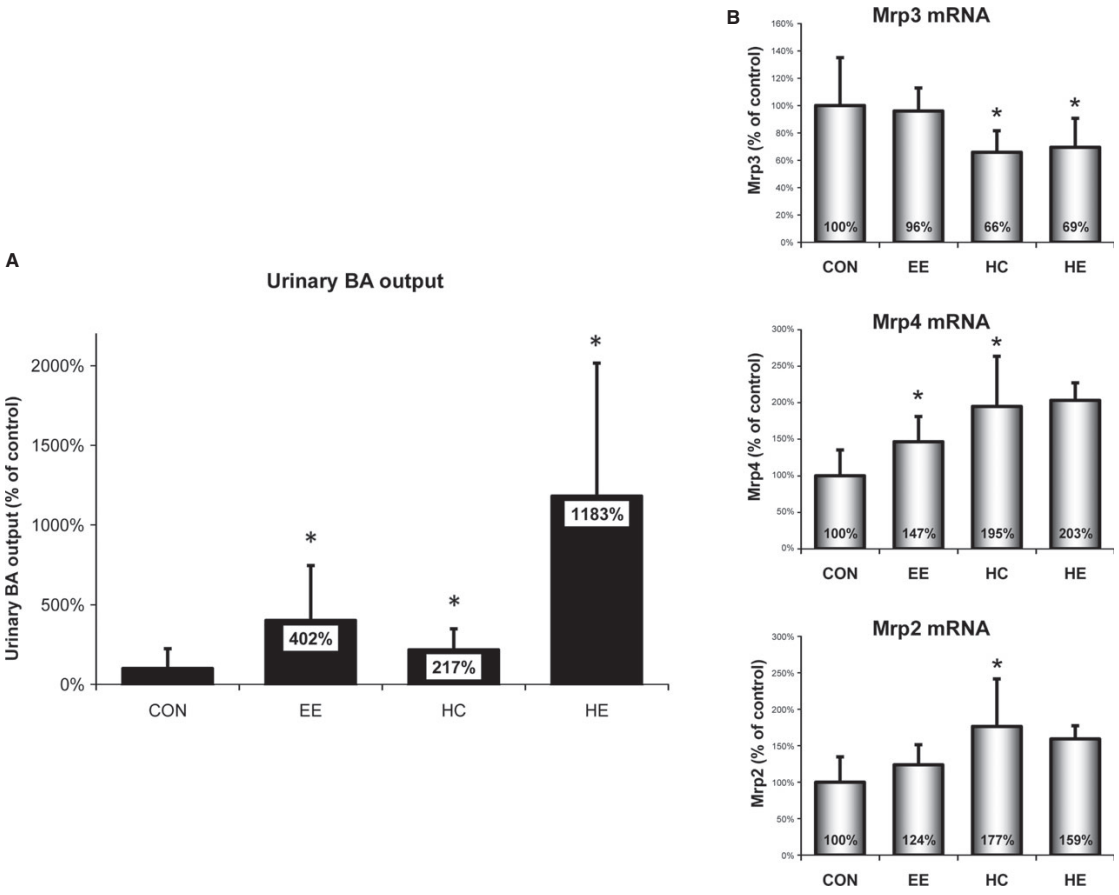

Fig. 4 Urinary bile acid (BA) output and mRNA expression of renal BA transporters. (**A**) BA concentration was measured in the urine collected for 30 min. from control (CON), ethinylestradiol (EE), heme (HC), or heme + EE (HE)-treated animals and expressed as relative changes from CON. (**B**) Relative expression of key renal BA transporters was measured in kidneys of CON, EE, HC, or HE-treated animals. $*P \leq 0.05$ *versus CON*.

hepatocytes revealed opposite effects of BA and EE on HMOX activity. While taurocholic acid was found to be a potent HMOX inhibitor, prolonged treatment with EE resulted in significant increases in HMOX activity. Thus, we speculated that the observed unaffected HMOX activity following EE-induced cholestasis might be as a result of an interaction of the opposing effects of BA and estrogens.

Hepatoprotective effect of HMOX has been described earlier in an ischaemia–reperfusion injury, graft-versus-host reaction or sepsis [25, 26, 37]. The hepatoprotection is believed to be conferred *via* HMOX metabolic products CO and biliverdin/bilirubin. While bilirubin protects the liver from oxidative stress triggered by high concentrations of BA [32], CO might have an effect on bile flow [24, 27, 28]. Heme has been long considered strong pro-oxidant with harmful effect on various organ systems. However, its ability to induce HMOX1 and form biologically active products has recently been implicated in beneficial effects in various experimental models including inflammatory bowel disease [38], diabetes [39], non-alcoholic liver disease [40], arterial hypertension [41] or sepsis [42]. In this study, we induced HMOX1 with heme applied in the form of methemalbumin to diminish the toxicity of free heme and increase formation of HMOX products.

In EE-treated animals, HMOX1 induction had clearly anti-cholestatic effect as measured by serum cholestatic markers. To understand this protective mechanism, we measured bile flow as well as biliary output of BA and glutathione, markers of bile salt-dependent and -independent bile flow, respectively. All these parameters were significantly increased in the heme pre-treated cholestatic group as compared with cholestasis without heme pre-treatment, although still much lower than in control group. A very similar pattern has been observed in the expression of key hepatic sinusoidal (*Oatps*, *Ntcp*)

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and canalicular (*Mrp2*) transporters, which were transcriptionally repressed in EE-treated, but to a much lesser extent in the heme pre-treated cholestatic groups. Recently, we have shown that inhaled CO can affect the expression of hepatic transporters [30], suggesting that CO generated in the HMOX pathway can contribute to an increase in the expression of liver transporters. It is important to note that the administration of heme resulted in an increase in total serum bilirubin levels in both control and cholestatic groups. In this case, we cannot consider bilirubin a cholestatic marker as its elevation in the serum was probably because of an increased formation arising heme degradation rather than the impaired clearance. Taken together, it appears that HMOX1 activation by heme can increase, but not normalize, both bile salt-dependent and -independent bile flow in cholestatic livers.

The only transporter specifically activated by heme was Mrp3. As described previously, Mrp3 is considered one of the basolateral overflow pumps compensating for impaired canalicular Mrp2 [8, 43]. Interestingly, HMOX1 activation by heme is mediated *via* Nrf2, a transcriptional factor responsible for activation of many antioxidative stress genes [44]. Moreover, transcriptional regulation of *Mrp3* by Nrf2 [45], and a possible anti-cholestatic effect of this pathway in mice [46], has been suggested recently. In our study, we observed a significant increase in *Mrp3* in all heme and/or cholestatic groups with the highest increase when both cholestasis and heme were present (HE group). Parallel *in vitro* experiments with primary rat hepatocytes confirmed key role of Nrf2 in heme-mediated Mrp3 overexpression. Heme pre-treatment with/without TCA increased *Mrp3* expression, while Nrf2 silencing repressed both basal and stimulated expressions of *Mrp3*.

Despite high *Mrp3* levels, we observed normal plasma BA concentrations in both heme-treated groups (HC and HE). The fact that Mrp3 transports BA from hepatocytes to plasma for renal excretion prompted us to focus on renal clearance of BA. As expected, we found an increase in urinary BA output in cholestatic animals. More importantly, heme was able to enhance urinary BA clearance both in CON and especially in EE-treated animals. Unlike in the liver, heme was able to promote adaptive renal transporter changes by increasing transporters responsible for renal clearance (*Mrp4*, *Mrp2*) and decreasing those for renal BA reabsorption (*Mrp3*) [47]. Tissue-specific differences in regulation of renal and liver transporters have been described earlier in obstructive cholestasis [48]. In accordance with these data, we have observed a marked reduction in liver *Mrp2* expression with its concomitant slight elevation in the kidney following EE administration and even more pronounced increase after heme pre-treatment. Accordingly, regulation of *Mrp3* expression by heme seems to be tissue-specific as well. While up-regulated in the liver, heme led to a significant decrease in its expression in the kidney (Fig. 5).

There are some limitations of our study. To assess the exact contribution of HMOX and/or heme signalling as anti-cholestatic

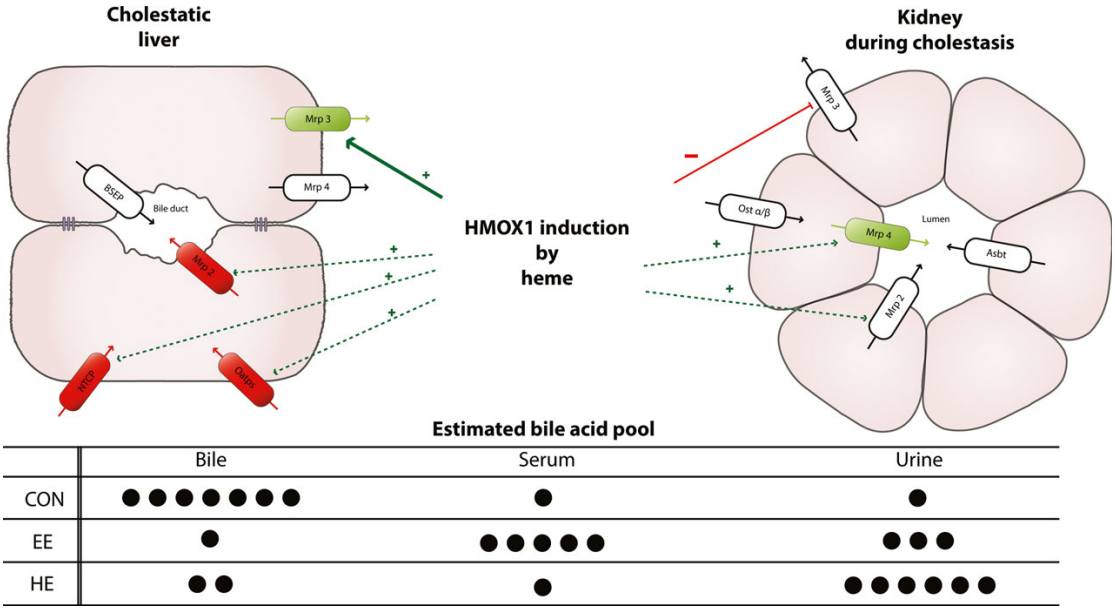

Fig. 5 Proposed mechanism of heme oxygenase-1 (HMOX1) induction on liver and kidney transporters in EE-induced cholestasis. EE-induced cholestasis is characterized by either decrease (red ovals) or increase (green ovals) in the expression of key hepatocyte and kidney transporters. Induction of HMOX1 with heme increased (green arrows), brought close to CON values (dashed green arrows) or decreased (red arrows) expression of these transporters. The changes in the expression of liver and kidney transporters result in the redistribution of bile acid pool in cholestatic (EE) and heme pre-treated (HE) animals.

agents, studies with HMOX1 knockout animals should be performed. However, to our knowledge, HMOX1 knockout rats are not available and mice do not develop cholestasis after EE administration and thus cannot be used for this type of experiments. Secondly, further studies should be performed to clarify the mechanism of heme-induced Mrp3 downregulation in rat kidney and also the putative effects of heme administration on BA synthesis and its enterohepatic circulation. Lastly, studies on different animal species and also clinical studies in human are needed to further confirm the feasibility of this approach to treat estrogen-induced cholestasis in humans.

We conclude that the induction of HMOX1 by heme increases expression of hepatocyte membrane transporters, subsequently stimulating bile flow in cholestatic rats. Moreover, heme stimulates hepatic expression of Mrp3 *via* a Nrf2-dependent mechanism. Conjugated BAs, transported by Mrp3 to plasma, are efficiently cleared by the kidneys resulting in normal plasma BA levels. Thus, the HMOX1 induction might represent a potential therapeutic strategy for the treatment of estrogen-induced cholestasis.

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Conflict of interest

The authors declare that they do not have anything to disclose regarding funding from industries or conflict of interest with respect to this manuscript.

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4 DISCUSSION

In presented papers, we demonstrated the important role of the heme catabolic pathway in hepatic disorders, in particular cholestasis and oxidative stress-induced liver injury, and the possible relationship of Hmox pathway regulation to the protection against liver damage.

In the first publication entitled „**Hepatoprotective effect of curcumin in lipopolysaccharide/D-galactosamine model of liver injury in rats: Relationship to HO-1/CO antioxidant system**“ we found that the *hmox1* upregulation by curcumin pre-treatment protected the liver against damage induced by LPS/DG (which serves as an experimental model for an apoptotic acute liver failure) [140]. The hepatoprotection was clearly demonstrated by the significant decrease in serum aminotransferase activities (ALT, AST) after curcumin pre-treatment in LPS/DG-induced toxicity. Furthermore, curcumin pre-treatment in the LPS/DG group was accompanied by significant increases in Hmox activity, *hmox1* expression and serum bilirubin levels contributing significantly to the hepatoprotective effect of curcumin. These results are in accordance with the reported data describing antioxidant properties of bilirubin [66, 145] together with our work [143], showing that intracellular accumulation of bilirubin can protect the liver from oxidative stress-mediated injury. The role of curcumin in hepatoprotection is supported by a decrease in lipid peroxidation measured as the production of conjugated dienes, nitrate production and total liver CO level as well as the downregulation of *NOS2* gene expression in curcumin pre-treated group. Curcumin alone did not affect Hmox activity or NOS-2/NO system but amplified the LPS/DG-induced *hmox* activation suggesting the important role of oxidative stress on the induction of Hmox/NOS system and its different mechanisms under pathophysiological conditions [146].

In accordance with our study, it was shown that the effect of curcumin on NO production was mediated via *hmox1* upregulation inactivating NF-κB *in vitro* [147]. Thus, we concluded the curcumin effect on the NOS-2/NO system downregulation and the decrease in accumulation of lipid peroxidation products *in vivo* was mediated via the Hmox system.

In our other study, we used the model of LPS-induced liver injury to answer the question if the modulation of the heme oxygenase pathway could specifically alter the cholestatic effects of LPS. CO is well known for its role in the regulation of inflammation, bile flow and liver integrity, however, its effect in early-phase of LPS-induced cholestasis has not been described. The results are recorded in the study entitled „**Protective effects of inhaled carbon monoxide in endotoxin-induced cholestasis is dependent on its kinetics**“ [141]. In accordance with our previous results on the protective effects of Hmox activation in LPS-induced liver injury we found that CO pre-treatment before LPS administration significantly decreased hepatocellular injury, as evident by decrease of AST activities, as well as markers of cholestasis (ALP, BAs), indicating that CO possesses important hepatoprotective properties. To further clarify the mechanism of this effect, we were interested in selected cytokines as they were previously proposed to be the cause of LPS-induced cholestasis [148]. We found that CO pre-treatment upregulated the expression of anti-inflammatory cytokine *IL10* that is in agreement with other studies demonstrating the anti-inflammatory effects of CO [37]. Simultaneously, there was a significant downregulation of LPS-induced *TNF* expression at 1h after CO pre-treatment. The similar mechanism of TNFα and IL-10 reciprocal regulation was described in another study [149] and might explain our results within 1 h of CO treatment when the concentration of CO in the liver was still elevated.

Since inflammatory cytokines can influence the expression of hepatic transporters [150], we investigated if there is an effect of CO pre-treatment on selected transporters on the basolateral and canalicular membranes. LPS treatment led to downregulation of mRNA expression of *Slc10a1*, *Abcb2* and *Slco1a1*, for transporters Ntcp, Mrp2, Oatp1a1, respectively, in time-dependent manner, and expression of *Abcb11* for transporter Bsep in 1 hr after LPS administration. Interestingly, these effects were partially prevented by administration of CO. The major effect on mRNA expression of *Abcb11*, *Abcc2* and *Slc10a1* was observed within 1 h with a persisting moderate effect of CO pre-treatment lasting for 4 h. Since CO half-life in the liver is very short and after 1 h from inhalation the half of CO is eliminated, the actual CO concentration and protective effect of CO on transporter expression seem to be linked. Unfortunately, in this study we were not able to answer the question if this early transient protective effect on hepatic transporter expression followed by a later decrease in serum cholestatic markers, can protect from long-term liver damage or further disease development. Further studies to measure late phase responses to single dose of CO prior to LPS exposure could clarify this question. In conclusion, we demonstrated that CO exposure substantially attenuated endotoxin-induced cholestasis in the manner that might be connected to inflammatory cytokines and subsequent hepatic transporters expression. This effect seems to be directly related to the kinetics of inhaled CO (Fig.8).

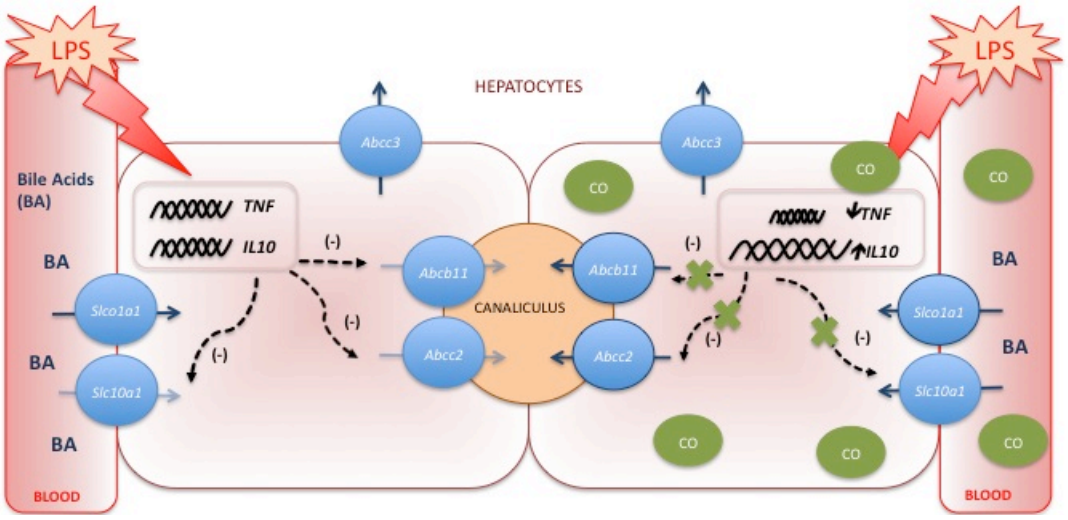

Fig.8. CO prevents the cholestatic effect of LPS*. LPS administration leads to upregulation of pro-inflammatory cytokines and subsequent downregulation of important hepatic transporters causing the accumulation of BAs in the hepatocytes and bloodstream. Administration of CO partly reverses effects of LPS on cytokine and hepatic transporter expressions. CO effects seem to be associated with its in vivo kinetics [141].*

Even though the protective effects of CO are well known, the kinetic profile and the ability to reach specific organs after its external delivery has not been previously described. For the first time, we characterized the kinetic profile of inhaled CO and reported the results in the study „**Protective effects of inhaled carbon monoxide in endotoxin-induced cholestasis is dependent on its kinetics**“ [141]. Furthermore, we established the detailed CO elimination profiles for different organs and kinetic parameters for rat. We found that both CO concentration and elimination half-lives were tissue dependent with biphasic elimination curves in all studied tissues. Maximum CO levels after its inhalation were found in blood (111-fold to control) while only slight changes were found in the brain (4-fold). Mean half-life values ranged from 0.6 h for the lungs and liver to 2.3 h for blood. Indeed other studies showed that the even CO

elimination half-life in blood differs significantly for different animal models [151, 152]. Altogether, these data indicate the necessity of carefully designing studies with CO application.

The role of bilirubin, another product of the heme catabolic pathway, in cholestatic diseases was investigated in the study entitled „**Bile acids decrease intracellular bilirubin levels in the cholestatic liver: implications for bile acid-mediated oxidative stress**“ [143]. While we found elevated markers of cholestasis (BAs, ALP and bilirubin) in 5 days after bile duct ligation (BDL) we simultaneously measured increase in the anti-oxidative capacity of plasma. As previously reported by Granato et al. [145], bilirubin displayed anti-oxidative effects in freshly isolated hepatocytes. Indeed in our study, bilirubin levels positively correlated with plasma antioxidant capacity. Interestingly after BDL, the bilirubin levels in plasma increased more than 600-fold but only 15-fold in the liver suggesting different metabolism under pathological conditions. In Gunn rats, which have a defect in bilirubin metabolism and the levels of unconjugated bilirubin are constantly increased, we even observed a marked drop in liver bilirubin after BDL. Thus in this model of extrahepatic cholestasis we showed for the first time that intracellular bilirubin is consumed during cholestasis and that plasma bilirubin levels might not reflect tissue bilirubin metabolism.

We were interested in the possible mechanisms underlying the bilirubin decrease in the liver tissue. Obstructive cholestasis - most likely high concentration of BAs - led to an increase in lipid peroxidation in our animal model, which is in agreement with other studies [153]. We found that *in vitro* taurocholic acid (TCA) downregulated the expression and activity of Hmox and as well lowered intracellular bilirubin levels. Moreover, adding TCA to liver homogenate increased lipid peroxidation in dose-dependent manner while adding TCA in liver homogenate from Gunn rats did not cause

lipid peroxidation. Adding bilirubin to the homogenate abolished the oxidative effect of TCA in liver homogenate, indicating the opposite effect of bilirubin and TCA on oxidative stress. In conclusion, even though bilirubin is an antioxidant, its concentration in the cholestatic liver can be depleted by high concentration of BAs. Importantly, high ratio of BAs/bilirubin might be implicated in the pathogenesis of oxidative liver damage in cholestasis.

Since we reported that bilirubin displays anti-oxidative effects in BA-stimulated oxidative stress, we extended our research on protective effects of UCB in human hepatoblastoma cells (HepG2) and rat liver cells exposed to other oxidative stressors and LPS, with focus on possible mechanisms responsible for the regulation of intracellular UCB levels. We concluded our results in the paper entitled „**Intracellular accumulation of bilirubin as a defense mechanism against increased oxidative stress**"[144]. According to our results, there is an equilibrium in intracellular and extracellular UCB levels in cells, which can be disrupted by oxidative insults. We found a significant accumulation of UCB in the liver tissue and serum of heme-treated animals. Furthermore, we showed for the first time that intracellular UCB levels in HepG2 cells were significantly elevated after *hmox1* induction with a low dose of oxidative stressor $NaAsO_2$ while total cellular heme was unchanged or rather increased, suggesting the upregulation of the heme biosynthetic pathway. Indeed, we confirmed *in vivo* that in LPS-treated rats where *hmox1* was upregulated, mRNA for the key enzyme in heme biosynthesis, 5-aminolevulinic acid synthase, was also upregulated, accompanied by an elevation in liver and serum UCB.

We found that heme-mediated *HMOX1* induction in HepG2 cells led to an elevation in intracellular UCB, which was associated with a dramatic decrease in lipid peroxidation. Furthermore, we measured lower accumulation of UCB and higher degree

of lipid peroxidation in the liver of rats with LPS-induced cholestasis. The treatment of HepG2 with sodium azide or TCA led to a dramatic depletion of intracellular UCB. This depleting process was completely abolished by increased production of UCB by HMOX or attenuated under hyperbilirubinemic conditions.

To conclude, the balance between intracellular and extracellular UCB levels can be disturbed by different oxidative stimuli. Depletion of intracellular UCB can decrease cytoprotection that might be prevented by *HMOX1* induction or systemic hyperbilirubinemia suggesting the potential pharmacological role of *HMOX1* inducers and/or UGT1A1 inhibitors. We hypothesize that UCB accumulation after heme supplementation might be the mechanism contributing to protection in various syndrome including dextran sulfate-induced colitis [154] or cadmium-induced testicular damage [155]. Furthermore, downregulation of *ABCC2* transporter during cholestasis might be a systematic protective response in an effort to maintain intracellular UCB levels under cholestatic conditions rather than simple consequence of liver dysfunction.

As products of the heme catabolic pathway showed an evident protective role in obstructive and sepsis-induced cholestasis we further focused on studying the role of *hmox1* induction in ethinylestradiol (EE)-induced cholestasis, representing the intrahepatic cholestasis of pregnancy, a rather frequent condition with unknown pathogenesis. In study entitled „**Protective effect of heme oxygenase induction in ethinylestradiol-induced cholestasis**“ we found that *hmox* induction by heme increased hepatocyte transporters expression and subsequently stimulated bile flow in cholestasis resulting in normal plasma BAs levels, and thus could confer to protection against EE-induced cholestasis [142].

Even though we previously reported that BAs downregulated *hmox1* expression and decreased Hmox activity with subsequent increase in oxidative stress and liver

injury in cholestasis we did not measured any changes in Hmox activity in our animal model. Thus we measured the effect of BA and EE directly on primary rat hepatocytes and found their opposite effects on Hmox activity. While TCA was found to be a potent inhibitor, prolonged treatment with EE caused significant increase in Hmox activity. In our model, seemingly unaffected Hmox activity might actually represent BA-suppressed *hmox1* induction, masking the protective effects of Hmox. Indeed, after *hmox1* induction by heme in EE-treated rats we found significant anticholestatic effects. Heme, usually presented as harmful pro-oxidative molecule, has been shown to be beneficial in several diseases as inflammatory bowel disease [154], diabetes [156] or sepsis [157]. In our study, heme treatment significantly decreased serum cholestatic markers and increased biliary output of BA and glutathione in EE-treated animals.

The mechanisms involved in EE-induced cholestasis are multifactorial and include downregulation of the main hepatocyte transporters - sinusoidal *Slc10a1* and *Slco* family [158, 159] and canalicular *Abcc2* and *Abcb11* [106, 160]. Comparably, a similar effect on *Slc10a1*, various *Slco*, and *Abcc2* was observed in EE-treated animals, but was partly prevented by heme pre-treatment. That corresponds with our previous results on prevention against downregulation of hepatic transporters by CO.

However, there was a significant upregulation of *Abcc3* by heme. Mrp3 is considered one of the basolateral overflow pumps compensating for impaired canalicular Mrp2 [106, 161]. Recently, *Abcc3* upregulation has been suggested a possible anticholestatic pathway in mice [162]. This transporter is regulated by Nrf2 [163] which is a transcriptional factor responsible for activation of many anti-oxidative stress genes [164] including *hmox1*. We confirmed on primary rat hepatocytes that Nrf2 is the key target in heme-mediated *Abcc3* overexpression. Since we did not find elevated BA levels in plasma of heme-treated animals as expected following *Abcc3*

upregulation, we decided to investigate renal clearance of BA. In both EE- and heme-treated animals the upregulation in renal clearance transporters (*Abcc2*) and downregulation of renal BA reabsorption transporter (*Abcc3)* was found. These data suggest the tissue-specific differences in the regulation of renal and liver transporters by heme and BAs, which is in agreement with the previous study on transporters in obstructive cholestasis by Lee et al. [165]. To conclude, the induction of *hmox1* expression and Hmox activity by heme regulates the expression of renal and hepatic transporters securing the bile flow in cholestatic rats. Heme stimulates hepatic expression of *Abcc3* via Nrf2, which leads to the effective clearance of BA into plasma and subsequently by the kidneys to urine.

5 SUMMARY

While heme seems to be a controversial compound, usually presented as a deleterious pro-oxidative molecule, otherwise showing beneficial effects in several diseases, the heme catabolic pathway is largely connected to the protection of cells from harmful insults. Heme is converted by HMOX into equal amount of CO, ferrous ion and BVR that is subsequently transformed into bilirubin by BLVRA. Despite the original thought that these products are only toxic waste products, the beneficial and signaling actions have been extensively documented. CO displays anti-inflammatory, antioxidant and vasoactive actions, while bilirubin is strong antioxidant with antiproliferative and anti-inflammatory effects.

In the presented thesis, we investigated the role of heme catabolic pathway in liver diseases, specifically in the modulation of inflammatory and cholestatic pathways. The liver is amongst the first organs confronted with many harmful insults, including bacterial endotoxin. We have shown that some anti-inflammatory agents, such as curcumin, protected the liver from damage via the increase in heme oxygenase activity and subsequent suppression of the pro-oxidative processes. Furthermore, exogenous administration of CO altered the expression of both anti-inflammatory and inflammatory cytokines in the liver of endotoxin-challenged animals leading to changes in expression of hepatic transporters and to decrease in markers of both hepatocellular injury and cholestasis. We extended our research on CO with the full kinetic profile *in vivo* which has not been previously described.

As bilirubin is known for its anti-oxidative effects we investigated its role in the model of obstructive cholestasis that is connected to oxidative injury caused largely by accumulation of BAs. The experiments revealed that the level of intracellular bilirubin was very important in protecting the liver from oxidative damage and was not reflected

by total plasma bilirubin level. Intracellular bilirubin was rather consumed during oxidative insults while BAs-inhibition of Hmox activity prevented the bilirubin production. Evoking hyperbilirubinemic conditions either by bilirubin supplementation or *hmox1* upregulation led to important hepatoprotection.

Furthermore, we described Hmox activity in liver tissue under cholestatic conditions caused by the administration of estrogens, which is the model of intrahepatic cholestasis of pregnancy. Seemingly unaffected Hmox activity could be the result of contradictory effects of elevated BAs and estrogens. In fact, the downregulation of hepatic transporters resulting in cholestasis was partly prevented by heme administration. We discovered that Hmox activation by heme regulated the expression of renal and hepatic transporters resulting in alternative BAs clearance into plasma and subsequently to urine by the kidneys.

To conclude, we discovered important involvement of heme and HMOX in cholestatic and inflammatory pathways in the liver. Our results suggest not only an important hepatoprotective role of the products of the heme catabolic pathway, but reflect the importance of maintaining the levels of intracellular bilirubin, which might represent an important pharmacological target. The regulation of HMOX and UGT1A1 activities might represent the therapeutical strategy for treatment not only cholestatic diseases, but also various disorders connected to oxidative stress and inflammatory processes.

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7 ABBREVIATIONS

Abcb11	gene for Bsep
Abcc2/3/4	gene for Mrp2/Mrp3/Mrp4
AP-1	activator protein 1
ASBT	apical sodium-dependent bile acid transporter
BAs	bile acids
Bach1	BTB and CNC homology 1 or basic leucine zipper transcription factor 1
BLVRA	biliverdin reductase
BSEP/Bsep	bile salts export pump, human/rat
BVR	biliverdin
CO	carbon monoxide
CO-RM	carbon monoxide-releasing molecule
DG	D-galactosamine
EE	ethinylestradiol
ER	endoplasmic reticulum
ERK	extracellular signal-regulated protein kinase
HCC	hepatocellular carcinoma
HCs	hepatocytes
HMOX/Hmox	heme oxygenase, human/rat
HSc	hepatic stellate cells
IBABP	ileal bile acid binding protein
IL	interleukin
iNOS	inducible nitric oxide synthase
IRAK	interleukin-1 receptor associated kinase
JNK	c-Jun N-terminal kinase
Keap1	Kelch-like ECH associating protein 1
KCs	Kupffer cells
Maf	transcriptional factor Maf
MAPK	mitogen-activated protein kinase
MRP/Mrp	multidrug resistance-associated protein, human/rat
MyD88	myeloid differentiation factor 88

NADPH	nicotinamide adenine dinucleotide phosphate
NF-κB	nucelar factor-kappa B
Nrf2	nuclear factor erythroid 2-related factor 2
NTCP/Ntcp	Na+-taurocholate cotransporting protein, human/rat
OATP/Oatp	organic anion transporting polypeptide, human/rat
OST	organic solute transporter
PI3K	phosphatidylinositol 3-kinase
PRRs	pattern-recognition receptors
REs	response elements
ROS	reactive oxygen species
SEc	sinusoidal endothelial cells
sGC	soluble guanylate cyclase
Slco10a1	gene for Ntcp
Slco	gene for Oatp
STAT	signal transducers and activators of transcription
StRE	stress-responsive element
TCA	taurocholic acid
TLR	toll-like receptor
TNF	tumor necrosis factor
UCB	unconjugated bilirubin
UGT1A1	bilirubin UDP-glucuronosyl transferase

,

8 LIST OF PUBLICATIONS

1) Muchova L,Vanova K, Suk J, Micuda S, Dolezelova E, Fuksa L, Cerny D, Farghali H, Zelenkova M, Lenicek M, Wong R, Vreman H, Vitek L. Protective effect of heme oxygenase induction in ethinylestradiol–induced cholestasis. J Cell Mol Med. 2015 Feb 16. doi: 10.1111/jcmm.12401 [Epub ahead].

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9 APPENDIX

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Adresát Katerina Vanova <Katerina.Vanova@lf1.cuni.cz>
Datum 17.03.2015 01:36

Dear Dr. Vanova,
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Best regards,

Jin-Lei Wang, Director, Editorial Office

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2015-03-17

Wang JL

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