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UNIVERZITA KARLOVA
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Indukce hemoxygenasy a biologická úloha jejích metabolických produktů.

Induction of heme oxygenase and biological role of its metabolic products

Disertační práce

Vedoucí závěrečné práce/Školitel: Doc. MUDr. Lucie Muchová, Ph.D.

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ABSTRAKT

Hemoxygenasa (HMOX) je enzym katalyzující první a rychlost limitující reakci štěpení hemu. Jejím působením vzniká oxid uhelnatý (CO), železnatý ion a biliverdin, který je následně redukován na bilirubin. CO byl před objevem mechanismu reakce katalyzované HMOX a ještě dlouho poté považován pouze za toxický produkt bez pozitivního významu pro lidský organismus. Podobně byl vnímán i bilirubin, marker jaterního poškození. Výsledky studií z posledních let ovšem ukazují, že HMOX i její metabolické produkty hrají významnou roli v řadě fyziologických procesů stejně tak jako i v obraně před procesy patologickými.

Cílem této práce bylo objasnit roli HMOX a jejích metabolicky aktivních produktů, především CO a bilirubinu, *in vivo* a *in vitro*. Zabývali jsme se studiem účinků CO, kdy jsme jako první popsali tkáňovou distribuci a farmakokinetiku inhalovaného CO u potkanů. Na modelu systémové sepse vyvolané endotoxinem u potkanů jsme zjistili, že inhalace CO je spojena s protizánětlivými a hepatoprotektivními účinky. U modelu cholestázy indukované ethinylestradiolem jsme prokázali anticholestatické účinky HMOX. Indukce HMOX1 jejím substrátem hemem zvyšovala expresi jaterních transportérů a tím podpořila tok žluče u cholestatických potkanů, zároveň usnadnila efektivní clearance konjugovaných žlučových kyselin ledvinami. V *in vitro* a *in vivo* studiích jsme prokázali, že CO inhibuje proliferaci buněk karcinomu pankreatu a je tak slibným potenciálním chemoadjuvantním agens v terapii tohoto obtížně léčitelného onemocnění.

Mírně zvýšené sérové koncentrace bilirubinu chrání organismus před oxidačním stresem a s ním asociovanými onemocněními. V silymarinu, extraktu z ostropestřce mariánského (*Silybum marianum)* jsme identifikovali některé flavonolignany, která jsou schopny *in vitro* a *in vivo* zvýšit tkáňové i systémové koncentrace nekonjugovaného bilirubinu a zároveň nevykazují hepatotoxické účinky. Silymarin je široce užívané hepatoprotektivum a indukce bilirubinu, významného endogenního antioxidantu, může být jedním z jeho mechanismů působení.

Výsledky této práce podtrhují důležitost úlohy HMOX a jejích produktů v metabolismu a naznačují nové možnosti terapeutického využití těchto látek.

Klíčová slova: hemoxygenasa, oxid uhelnatý, bilirubin, silymarin, UGT1A1, hepatoprotektivum, cholestáza

ABSTRACT

Heme oxygenase (HMOX) catalyzes first and rate-limiting step in heme degradation. By its action, carbon monoxide (CO), ferrous iron and biliverdin which is subsequently reduced to bilirubin are produced. Before discovery of HMOX reaction mechanism, CO was considered only a toxic waste product without any significant importance for human organism. Bilirubin, marker of liver dysfunction, has been also exposed to similar perception. But results from past decades show that HMOX and its metabolic products play an important role in number of physiological as well as defense against pathophysiological processes.

The aim of this thesis was to clarify the role of HMOX and its metabolic products, presumably CO and bilirubin, *in vivo* and *in vitro*. We focused on the role of CO in a rat model of lipopolysaccharide-induced cholestasis. We were first to describe tissue distribution and pharmacokinetics of inhaled CO in this animal model and found out that CO inhalation is associated with anti-inflammatory and hepatoprotective effects. In a rat model of ethinylestradiol-induced cholestasis, we demonstrated the anticholestatic effect of HMOX. The induction of HMOX by its substrate heme increased the expression of liver transporters thereby increasing bile flow and simultaneously facilitated effective clearance of conjugated bile acids by kidney in cholestatic animals.

In *in vitro* and *in vivo* studies we proved that CO inhibits proliferation of pancreatic cell lines suggesting a potential of CO use as a supportive measure against this serious type of cancer with limited therapeutical options.

Mildly elevated bilirubin concentrations in serum protect organism against oxidative stress and associated diseases. In silymarin, an extract from *Silybum marianum,* we have identified flavonolignans capable of *in vitro* and *in vivo* elevation of tissue as well as systemic concentrations of unconjugated bilirubin without hepatotoxic effects. Silymarin is a widely used hepatoprotectant and induction of bilirubin, important endogenous antioxidant, can be one of the mechanisms of its action.

Results of this thesis underline the important role of HMOX and its products in metabolism and indicate a new potential therapeutic use of these compounds.

Key words: heme oxygenase, carbon monoxide, bilirubin, silymarin, UGT1A1, hepatoprotectant, cholestasis

CONTENT

1 INTRODUCTION

1.1 Heme

Metalloproteins account for nearly half of all proteins in nature (Lu Y. et al., 2009) and heme proteins in particular belong to the most important ones due to their large array of biological functions. Heme is a complex of iron with protoporphyrin IX serving as a prosthetic moiety to numerous hemoproteins that are essential for the function of all aerobic cells (Ponka P., 1999). Hemoproteins are involved in a remarkable array of crucial biologic functions including oxygen binding (hemoglobin, myoglobin), oxygen metabolism (oxidases, peroxidases, and catalases) and electron transfer (cytochromes) (Lin Y.-W. & Wang J., 2013). Heme forms also a prosthetic group of proteins with important regulatory or signaling properties including guanylate cyclase, hydroxylases and nitric oxide synthase. Serving also as a regulatory factor, heme plays an important role in controlling the expression of numerous proteins including globin, heme biosynthetic enzymes, cytochromes, myeloperoxidase, heme oxygenase-1 and transferrin receptor (Ogawa K. et al., 2001; Ponka P., 1999). Cellular heme levels seem to be tightly controlled due to its prooxidative nature. This is achieved by a fine balance between heme biosynthesis and catabolism regulated by the enzyme heme oxygenase. Spectra of heme functions in mammal organism is wide from regulating either transcription and/or translation to processing, assembly, or stability of hemoproteins in nonerythroid cells (Ponka P., 1999).

1.2 Heme oxygenase

Heme oxygenase (HMOX) (E.C 1.14.99.3) is an oxidoreductases class enzyme catalyzing the first and rate-limiting step in the degradation of heme. HMOX system consists of HMOX and NADPH–cytochrome P450 reductase (Tenhunen R. et al., 1972).

Via oxidation, HMOX cleaves the α-meso carbon bridge of b-type heme molecules to yield equimolar quantities of biliverdin IXa (BV), carbon monoxide (CO), and free iron. BV is subsequently converted to bilirubin (UCB) via the action of biliverdin reductase (BVR), and free iron is promptly sequestered into ferritin (Fig 1.) (Otterbein L.E. & Choi A.M.K., 2000).

HMOX was originally identified in 1968 and 1969 by Tenhunen *et al.* when they characterized the enzyme HMOX as well as its cellular localization (Tenhunen R. et al., 1968, 1969). Initially, it was supposed that HMOX reaction involves cytochrome P450 as a terminal oxidase (Tenhunen R. et al., 1972). Studies from Yoshida *et al.* have shown that HMOX is independent of any type of cytochrome P450 (Yoshida T. & Kikuchi G., 1974; Yoshida T. & Kikuchi G., 1978; Yoshida T. et al., 1974).

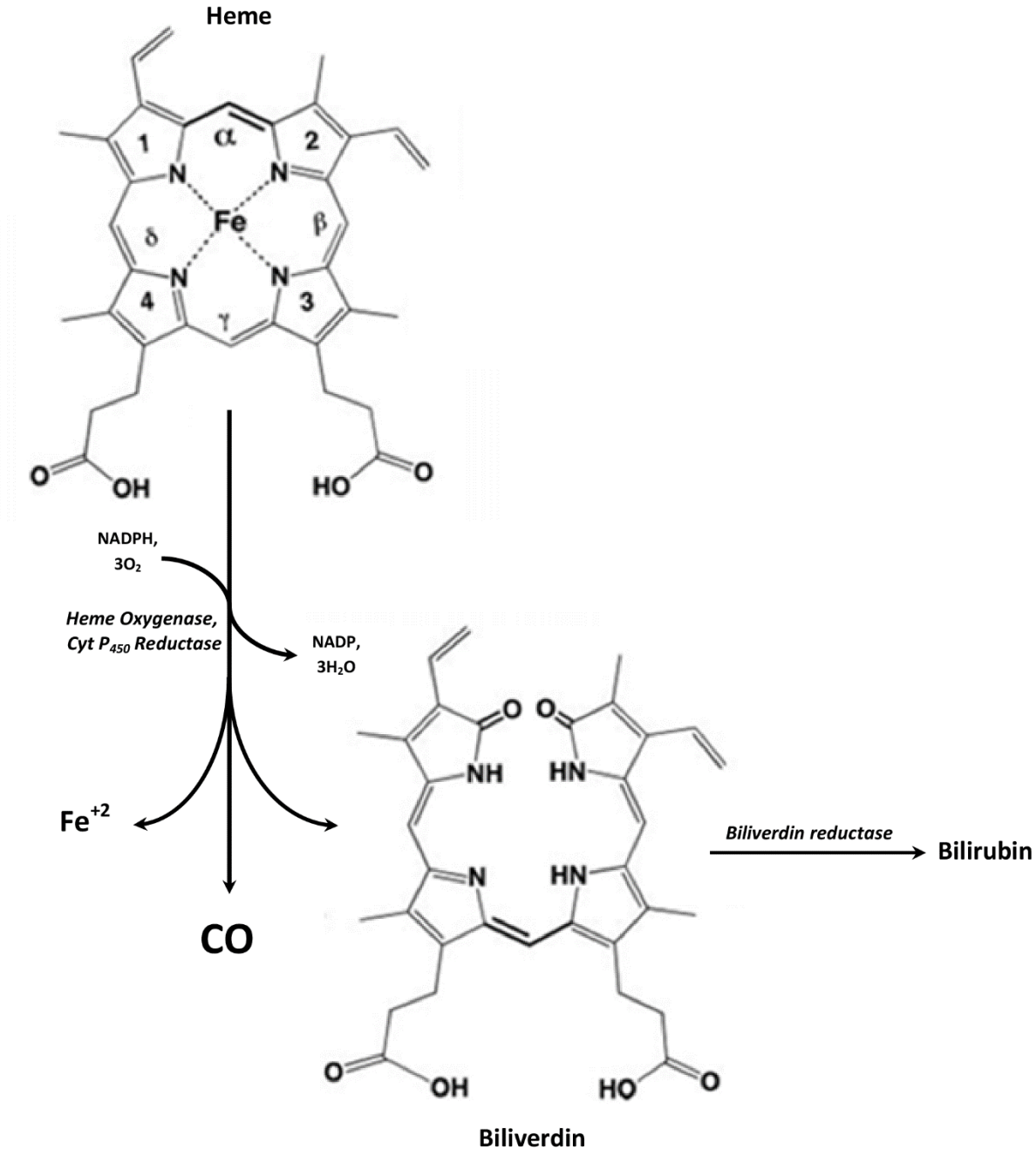

Fig 1. Heme degradation pathway (Šuk J. & Muchova L., 2018):

Heme oxygenase catalyzes a rate-limiting step of heme degradation. Oxidative degradation of heme molecule generates equimolar amount of CO, ferrous ion (Fe 2+) and biliverdin. Biliverdin is subsequently reduced by biliverdin reductase to bilirubin. The reaction requires 3 mol of O_2 NADPH:cytochrome P-450 reductase serves and as a source of electrons.

To this date, two enzymatically active isoforms of HMOX have been identified, a 33 kDa inducible HMOX1 and a 36 kDa constitutive HMOX2 (Maines M.D. et al., 1986). Both enzymes have hydrophobic sequences at their C-terminal ends that are involved in binding to microsomal membrane. Interestingly, a trypsin-treated HMOX lacking the C-terminal hydrophobic region is fully active and is water-soluble (Yoshida T. et al., 1991). Expression of truncated HMOX1 and HMOX2 in Escherichia coli helped a characterization of HMOX and an understanding of the reaction mechanism at the molecular level (Kikuchi G. et al., 2005).

Under physiological conditions, HMOX activity is highest in the spleen and specialized reticuloendothelial cells of the liver and bone marrow where senescent erythrocytes are sequestered and destroyed but its activity has also been observed in all systemic organs (Otterbein L.E. & Choi A.M.K., 2000; Ryter S. W. et al., 2006).

The highest expression of HMOX2 can be detected in the testes but the protein is also found in other systemic tissues including, but not limited to, the brain and central nervous system, vasculature, liver, kidney, and gut (Maines M.D., 1997; Maines M.D. et al., 1986; Trakshel G.M. et al., 1986; Zakhary R. et al., 1996). In the liver, HMOX1 is predominantly expressed in Kuppfer cells, while in hepatocytes is prevalent HMOX2 (Goda N. et al., 1998).

Heme is one of the HMOX1 inducers but several studies demonstrated that HMOX1 enzyme activity could also be stimulated by a variety of non-heme products including ultraviolet irradiation, endotoxin, heavy metals, and oxidants such as hydrogen peroxide (Keyse S.M. & Tyrrell R.M., 1989; Maeshima H. et al., 1996; Vile G.F. & Tyrrell R.M., 1993). Generation of reactive oxygen species followed by oxidative stress is one of the common feature of these inducers.

Based on these implications, HMOX1 is able to respond to oxidative stress in cells and serves as a cytoprotective molecule against it. Although the function of this enzyme is still incompletely understood, accumulating evidence strongly suggests that the endogenous induction of HMOX1 provides potent cytoprotective effects in various *in vitro* and *in vivo* models of cellular and tissue injury (Otterbein L.E. & Choi A.M.K., 2000; Otterbein Leo E. et al., 2003; Ryter S. W. et al., 2006; Shibahara S. et al., 2002; Willis D. et al., 1996).

The importance of HMOX is well documented in cases of HMOX1-deficiency HMOX1($^{-/-}$) in mice and humans. HMOX1($^{-/-}$) mice were first generated in 1997. Majority

of them do not survive to term, and the mice that do survive to adulthood are abnormal and die within one year of birth.

Adult mice exhibit growth retardation and normochromic, microcytic anemia. Kidneys and livers from these mice show evidence of iron deposition, and as these HMOX1($^{-/-}$) mice age, they also demonstrate an increased presence of chronic inflammation characterized by hepatosplenomegaly, leukocytosis, glomerulonephritis, and hepatic periportal inflammation. It has been described that cells obtained from these mice are more susceptible to oxidative stress induced by endotoxin (Poss K.D. & Tonegawa S., 1997a, 1997b). The first known human case of HMOX1($^{-/-}$) have been reported in 1999. Patient exhibited similar phenotypic alterations as those observed in the HMOX1($^{-/-}$) mice, including growth retardation, anemia, leukocytosis, and increased sensitivity to oxidative stress, supporting the importance of HMOX role in cytoprotection against oxidative stress (Yachie A. et al., 1999).

1.2.1 Biliverdin

Biliverdin (BV) is formed in a single reaction during catalysis of heme by heme oxygenases. Carbon monoxide and iron are also released at the same time. The rate of heme degradation in the reticuloendothelial organs (spleen and liver) is slow, therefore all BV is readily reduced to unconjugated bilirubin (UCB). BV is a green hydrophilic tetrapyrrolic bile pigment responsible for a greenish color sometimes seen in bruises (Wegiel B. & Otterbein L.E., 2012). The evolution of UCB from BV has been recognized since the studies of Lemberg in 1936 (Lemberg R. & Wyndham R.A., 1936) and confirmed by metabolic labeling experiments (Goldstein G.W. & Lester R., 1964). There are two isoforms of an enzyme BVR. BVR-A, which catalyzes conversion of BV-a specifically and is expressed in the majority of adult tissues and inducible with stress, and BVR-B which is present during embryogenesis and is an isoform specific for the BV-d and b isomers. BVR activity has a dual, pH-dependent, cofactor requirement, utilizing NADH at pH 6.7, but NADPH in the basic range (pH 8.7) (Kutty R.K. & Maines M.D., 1981; McCoubrey W.K., Jr. et al., 1995). Another unique feature is that autophosphorylation of BVR triggers an increase in its reductase activity, augmenting the production of bilirubin and thus enhancing defense against oxidative stress (Salim M. et al., 2001). Regulation of HMOX1 and BVR expression and their enzymatic activity are critical for the function of a heme degradation pathway.

BVR is present in excess, resulting in the absence of biliverdin in human plasma or bile (Crawford J.M. et al., 1988).

BVR expression is regulated negatively by nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) activation and positively by hypoxia-mediated factor 1 α (HIF1α) stabilization and specific hormone response element (HRE) binding sites in the BVR promoter (Gibbs P.E. et al., 2010). BVR is strongly induced by its substrate biliverdin as well as other agents that induce oxidative stress including lipopolysaccharide (LPS), heavy metals, and toxins (Maines M.D. et al., 2001; Wegiel B. et al., 2009). BVR not only generates powerful antioxidant converting BV to UCB, but seems to have other biologically important functions in cell signaling (Maines M.D., 2005). BVR exhibits the rare function of a multi-specific (serine/threonine/tyrosine) kinase contributing to cell signaling (Lerner-Marmarosh N. et al., 2005). Similar to HMOX, BVR can translocate from the cytosol into the nucleus, where it functions as an oxidative stress-induced transcription factor in a variety of signaling pathways; these include activator protein 1-regulated genes and transforming growth factor β (TGF-β), NF-κB, stress response including HMOX1, survival and Jak-Stat pathways (Kravets A. et al., 2004), as well as mitogen-activated protein kinases (MAPK) signaling pathway (Lerner-Marmarosh N. et al., 2008). BVR functions also as a cell membrane protein in macrophages where it mediates signaling of biliverdin through the phosphatidylinositol-4,5-bisphosphate 3-kinase/protein kinase B (PI3K/Akt) pathway and acts also as a negative adapter of toll-like receptor 4 (TLR4), suppressing pro-inflammatory cytokines (Vitek L. & Ostrow J.D., 2009; Wegiel B. & Otterbein L.E., 2012).

1.2.2 Bilirubin

UCB, the end product of the heme catabolic pathway, was discovered in 1847 by Rudolf Virchow. UCB molecule consists of four pyrrole rings (A - D) connected by carbon bridges. Molecule is nearly symmetrical and composed of two planar dipyrrole units (rings A-B and C-D) joined to each other by a saturated central methylene group (-CH2-). UCB is poorly stable, since the double bonds in the vinyl and methene groups are readily oxidized and highly photosensitive. The natural bilirubin species in humans is the UCB IX α 4Z,15Z molecule. Other UCB isoforms include III α and XIII α isomers formed by molecular scrambling, a nonenzymatic process in which UCB IX α is split into two halves which then randomly re-assemble (Fig 2) (Vitek L. & Ostrow J.D., 2009).

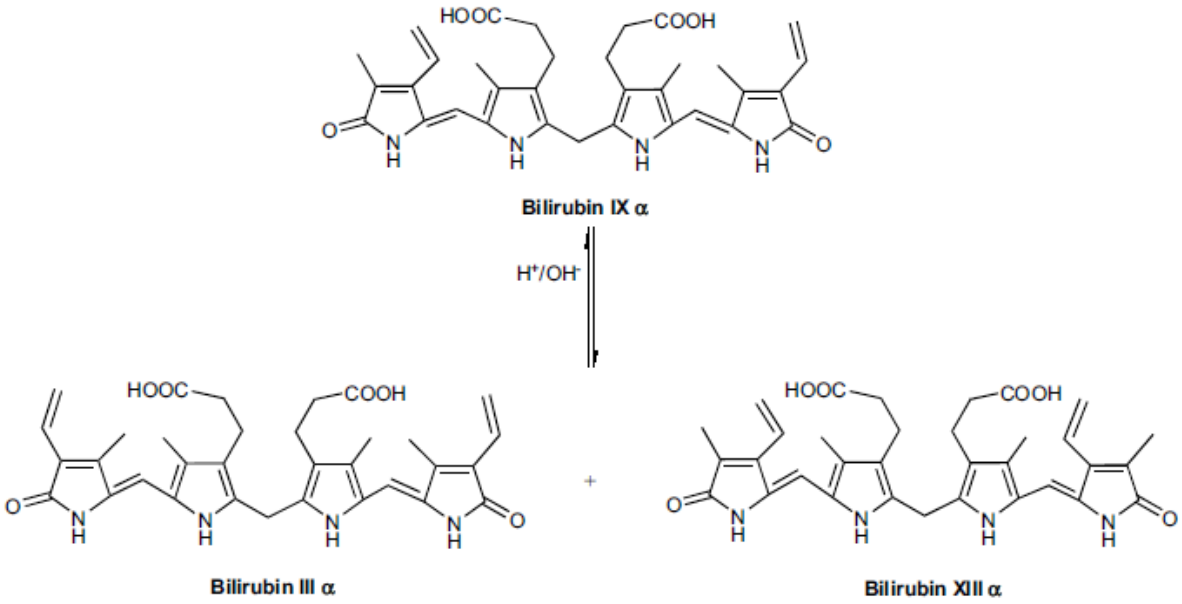

Fig 2: Bilirubin IX α and its constitutional isomers III α and XIII α formed by dipyrrole exchange (Modified from (Vitek L. & Ostrow J.D., 2009))

Since UCB is highly insoluble in water due to aninternal hydrogen bonding (Ostrow J.D. et al., 1994) of its polar groups which are hidden from interaction with water molecules (the solubility threshold in plasma is 70 nM) (Gazzin S. et al., 2017), it must be transported in plasma bound mainly to serum albumin (90%)(Jacobsen J., 1969) and secondary to the apolipoprotein D in high density lipoprotein (HDL)(10%)(Goessling W. & Zucker S.D., 2000; Suzuki N. et al., 1988). Under physiological conditions, only a very marginal part (<0.1%) of serum UCB is not bound to albumin or HDL and is termed “free UCB” (Bf)

which is the fraction that can diffuse into tissues and cause cytotoxicity(Calligaris S.D. et al., 2007). Out of 4.4 ± 0.7 mg/kg of UCB produced each day, 75% to 80% derives from hemoglobin released during destruction of senescent red blood cells in the reticuloendothelial system, and remaining 20 to 25% percent result from ineffective erythropoiesis and breakdown of muscle myoglobin and liver cytochromes (Vitek L. & Ostrow J.D., 2009).

The exact mechanism of UCB transport into cell is still a matter of discussion, evidence so far shows that minor part of UCB is transport by passive diffusion and a major part by an active transport mediated by organic anion transporting polypeptides (OATP) 1B1 and 1B3 (van de Steeg E. et al., 2012). The intracellular fraction of UCB among others contribute to protection of cellular lipids against peroxidation (Gazzin S. et al., 2016; Zelenka J. et al., 2012).

In hepatocytes, fat soluble UCB is converted to conjugated bilirubin (CB), in which one or both -COOH groups are modified by covalent attachment of polar groups, principally glucuronic acid by the action of a UDP-glucuronosyl transferase 1A1 isoform (UGT1A1). Glucuronide moiety disrupts the internal hydrogen bonding of the modified -COOH group, and the released polar groups of UCB plus the -COOH and multiple -OH groups of the glucuronide moiety now interact with water, forming the water soluble conjugated bilirubin (Vitek L. & Ostrow J.D., 2009).

CB is actively secreted against the concentration gradient through the canalicular membrane of hepatocyte into the bile by the ATP- binding cassette C2 (ABCC2) and, possibly by ABCG2 multidrug transporters (Kamisako T. et al., 1999). Under physiological conditions, prior its secretion into the bile, a part of CB is transported back to the blood by the action of the sinusoidal (basolateral) transporter ABCC3 and subsequently reuptaken into the hepatocyte by the organic-anion-transporting polypeptide (OATP) B1/B3. This mechanism is believed to be responsible for protecting the periportal hepatocytes from the excessive bilirubin and xenobiotics accumulation (van de Steeg E. et al., 2012). CB secreted into the bile is poorly absorbed from the small intestine and is almost all deconjugated by action of β-glucuronidases released from enterocytes and coliform bacteria. Part is excreted as UCB but the majority is reduced to urobilinogens (mainly urobilinogen and stercobilinogen) by coliform bacteria. A small fraction of urobilinogens undergoes enterohepatic circulation and are absorbed from the intestine to the portal tract and re-secreted by the liver into the bile or excreted into the urine by kidney. However, most of

them are oxidized to urobilins and stercobilin and excreted via faeces (Fig 3) (Tiribelli C. & Ostrow J.D., 2005).

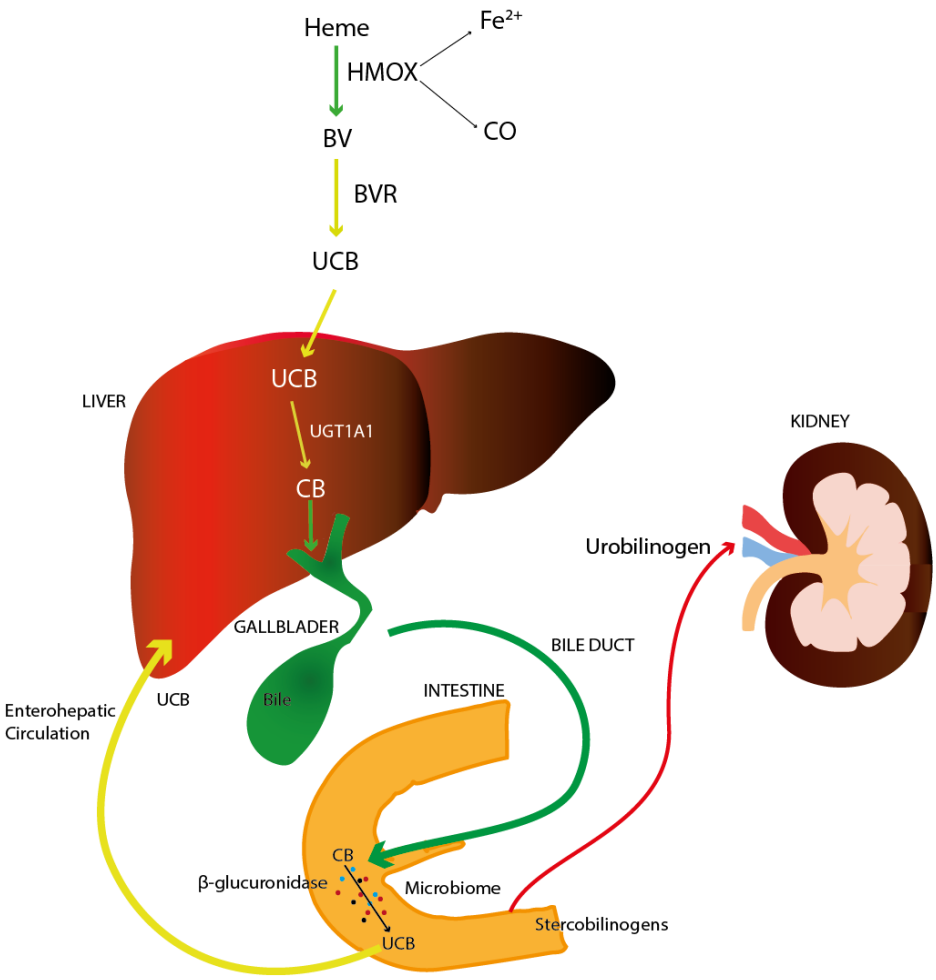

Fig 3: Bilirubin metabolism.

HMOX, heme oxygenase; BV, biliverdin; BVR, bilirubin reductase; UCB, unconjugated bilirubin; UGT1A1, UDP-glucoronosyl transferase 1A1; CB, conjugated bilirubin;

UCB has long been recognized as a marker of liver dysfunction or a potential toxic compound causing severe brain damage in newborns. But UCB is also an important antioxidant that represents 10% of blood antioxidant capacity (Belanger S. et al., 1997). The major change in understanding bilirubin role in organism occurred in 1987 when Roland Stocker and his colleagues showed 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 (Stocker R. et al., 1987).

Mildly elevated bilirubin levels in serum, typically seen in the Gilbert syndrome phenotype, were associated with a significantly lower prevalence of colorectal cancer and a

reduction in cardiovascular disease risk (Schwertner H.A. & Vitek L., 2008). Furthermore, low levels of serum bilirubin are considered to be a risk factor for development of cardiovascular diseases. It has been proven that each micromolar elevation of serum bilirubin decreases the risk of cardiovascular diseases by 6,5% (Novotny L. & Vitek L., 2003).

Bilirubin production is dependent on HMOX activity, but systemic bilirubin concentrations are predominantly affected by hepatic UGT1A1, its biotransforming enzyme (Lin J.P. et al., 2010). Partial inhibition of bilirubin glucuronosylation was proposed as a wise strategy for inducing "iatrogenic" Gilbert syndrome (McCarty M.F., 2007). This indeed was demonstrated as a "side effect" of several drugs used in various indications, typically for atazanavir, which administration is often associated with mildly elevated concentrations of unconjugated bilirubin. Surprisingly, hyperbilirubinemia induced by atazanavir was reported to decrease markers of oxidative stress (Estrada V. et al., 2014) and cardiometabolic risk factors (Milian J. et al., 2015), as well as improvement in endothelial functions (Dekker D. et al., 2011), but safer approaches using for example well characterized natural compounds are certainly needed.

UCB possesses also anti-inflammatory properties. They seem to be mediated via inhibition of NADPH oxidase, vascular cell adhesion protein 1 (VCAM-1) and inducible nitric oxide synthase (iNOS) reducing the trans-endothelial leucocyte migration as shown in animal models of inflammatory colitis (Zucker S.D. et al., 2015) and allergen-induced airway inflammation and asthma (Keshavan P. et al., 2005). Moreover, UCB is involved in the complex network of other signaling pathways, such as arylhydrocarbon receptor (Ahr) (Yeager R.L. et al., 2009), nuclear factor (erythroid-derived2)-like2 (Nrf2) (Qaisiya M. et al., 2014), protein kinase C (PKC) (Amit Y. & Boneh A., 1993) and many others (Fig 4)(Gazzin S. et al., 2016).

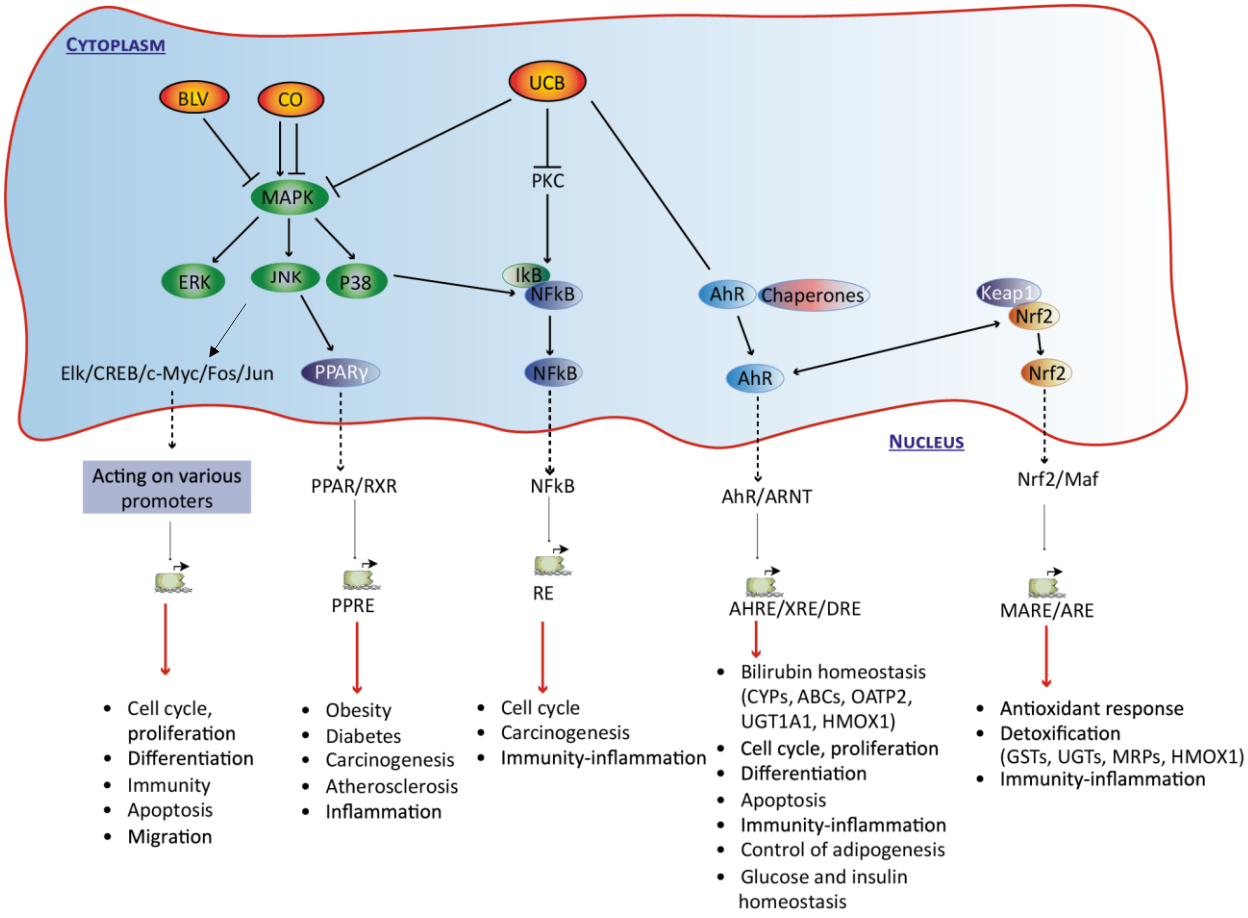

Fig. 4: Unconjugated bilirubin signaling pathways.

Unconjugated bilirubin (UCB), biliverdin (BLV) and CO acts on mitogen-activated protein kinases (MAPKs), p38, cJNK (Jun N-terminal kinases), ERK (extracellular signal-regulated kinases) pathway. Via inhibition of MAPKs pathway, it regulates an activation of the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kB) pathway, peroxisome proliferator-activated receptor (PPAR)-γ, Elk (ETS domain- containing protein), CREB (cAMP response element-binding protein) /c-Myc/c-Fos/Jun and their translocation into nucleus where there are subsequently acting on various promoters. NF-kB translocation into nucleus can be inhibited also via PKC pathway. UCB also stimulates arylhydrocarbon receptor (AhR) translocation in to the nucleus, interacting with ARNT (AhR nuclear translocator)/AHRE (Ahr responsive elements) / XRE (xenobiotic responsive element) / DRE (drug responsive element). There is also interconnection between AhR and Nrf2 [nuclear factor (erythroid-derived2)-like2] pathways; Keap1 - Kelch-like ECH-associated protein 1, MARE- MAf Recognition Element, ARE - Antioxidant Responsive Elements

1.2.3 CO

Carbon monoxide is a colorless, odorless gas, mostly know as a product of incomplete combustion of organic compound, and it is highly dangerous for its high affinity to hemoglobin followed by tissue hypoxia (Ryter S.W. & Otterbein L.E., 2004). But CO is also produced endogenously during the heme degradation (Tenhunen R. et al., 1969).

A change of perception in case of CO has occurred in 1993 when Verma *et al.* discovered that CO works as a neurotransmitter (Verma A. et al., 1993). The fact that CO is produced endogenously has been described already in 1949 by Swedish scientist Torgny Sjostrand (Sjöstrand T., 1949). Less than twenty years later, Raimo Tenhunen from the laboratory of Rudi Schmid discovered that CO is produced during heme degradation in a reaction catalyzed by HMOX (Tenhunen R et al., 1968). Recent study showed that CO through binding to heme proteins affects soluble guanylate cyclase (sGC), cytochromes p450, cytochrome c oxidase, iNOS and some intracellular signaling pathways with anti-inflammatory, anti-apoptotic anti-proliferative and anti-coagulative effect (Ahmad S. et al., 2015; Motterlini R. & Otterbein L.E., 2010; Otterbein L.E., 2002; Ryter S.W. & Otterbein L.E., 2004). Binding of CO to sGC leads to conversion of GTP to cGMP, which serves as a second messenger and has an important role in many physiological processes including aggregation of thrombocytes, fibrinolysis, regulation of vascular tonus and cell cycle or neurotransmission (Wang R. et al., 1997; Wu L. & Wang R., 2005). CO can affect intracellular signaling pathways also directly, for example MAPK responsible for cell growth and regulation of inflammatory response (Fig 5) (Otterbein L.E. et al., 2000).

Expression of both anti-inflammatory (IL-10) and pro-inflammatory (tumor necrosis factor α (TNFα), IL-1β, IL-6,) cytokines have been shown to be regulated by CO via different mechanisms in both *in vivo* and *in vitro* studies showing anti-inflammatory effects of CO (Morse D. et al., 2003; Otterbein L.E. et al., 2000). In cholestasis characterized by an impairment of bile formation and/or outflow, has been show that impairment of sinusoidal uptake and canalicular transport of bile acids is caused by down-regulation of the main membrane transporters as a response to action of pro-inflammatory cytokines (Geier A. et al., 2007; Trauner M. et al., 1998). This is one of the mechanism where CO can counteract (Chen C.-Y. et al., 2013).

CO has its indispensable role in liver and bile metabolism where it contributes to the maintenance of blood perfusion in the liver and to the excretion of bile (Suematsu M. et al., 1994). Also, it regulates the contractility of bile canaliculi by suppression of intracellular calcium mobilization (Shinoda Y. et al., 1998) and modulates the expression of liver transporters (Chen C.-Y. et al., 2013).

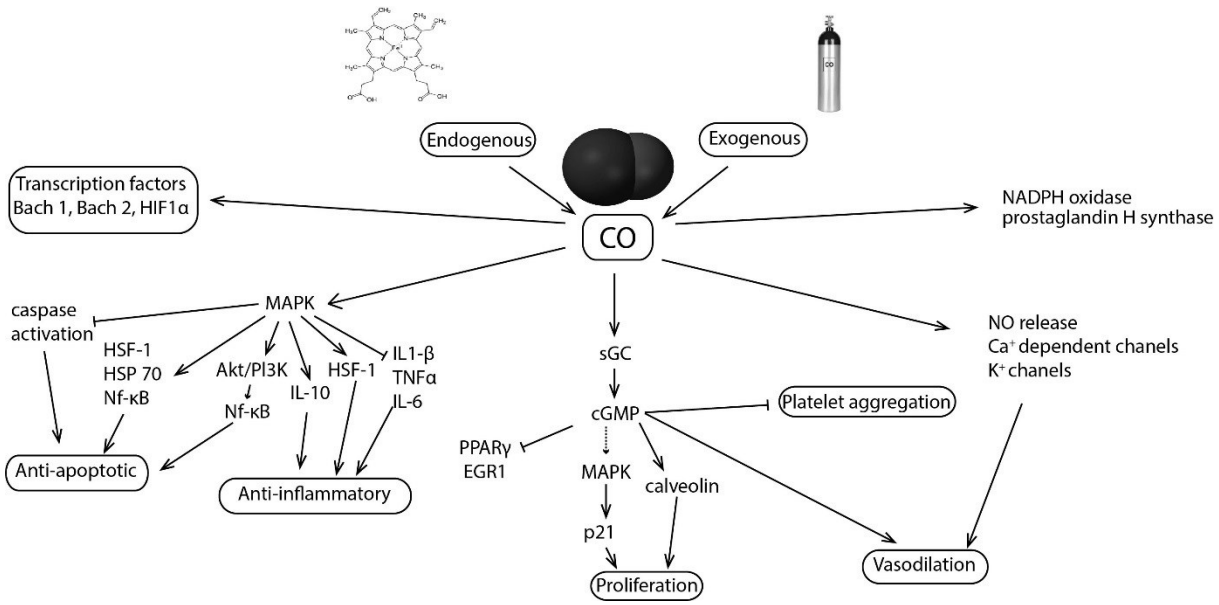

Fig 5: CO mechanism of action and molecular targets (Šuk J. & Muchova L., 2018)

CO produced endogenously or exogenously modulates apoptotic, inflammatory, proliferative, vasodilatory, aggregation and other cellular functions. Primary targets of CO are proteins with heme moiety such as soluble guanylate cyclase, cytochromes p450, cytochrome-c-oxidase, inducible NO synthase or NADPH oxidase. CO affects also transcription factors (Bach 1, 2; HIF1α) or mitogen-activated protein kinase (MAPK); HIF1α - Hypoxia-inducible factor 1- α, Akt - Transcription factor, Pl3K- Phosphatidylinositol-4,5-bisphosphate 3-kinase, HSF1 – heat shock factor 1, HSP 70 – heat shock protein 70, Nf-κB - nuclear factor kappa-light-chain-enhancer of activated B cells, IL-1β – interleukin 1 β, IL-6 – interleukin 6, IL-10 – interleukin 10, PPARγ - Peroxisome proliferator-activated receptor γ, EGR1 - Early growth response protein 1

Biological effects of CO have been studied since 1993 using in particular inhalation application of gaseous forms (Motterlini R. et al., 2002). However, inhalation of CO has its limitations, especially when majority of administered CO is bound to hemoglobin and only trace amount is distributed to the tissue of interest (Vanova K. et al., 2014). Because of that,

there is an effort to develop alternative ways of application of this protective compound. The alternative offers the use of carbon monoxide releasing molecules (CORM) which have been developed in recent years as experimental therapeutics (Motterlini R. & Otterbein L.E., 2010).

CO has shown its protective properties *in vivo* especially in a therapy of systemic inflammation (Knauert M. et al., 2013), liver and intestine inflammatory disease (Šuk J. & Muchova L., 2018), cancer treatment (Szabo C., 2016) or ischemia reperfusion (I/R) injury and organ transplant (Ozaki K.S. et al., 2012). There is an effort to move use of CO towards clinical practice but its dependent on result of currently running clinical trials (Šuk J. & Muchova L., 2018).

1.3 Hyperbilirubinemia

Physiological total serum bilirubin level varies within the range of 0.2- 1 mg/dL (3.4-17.1 μM), with UCB forming a predominant fraction and CB being a negligible part (Gazzin S. et al., 2017). While mildly elevated systemic bilirubin levels, typically seen in the Gilbert syndrome phenotype subjects (see below), are believed to have protective effects against oxidative stress related diseases (Vitek L. et al., 2002), highly elevated levels (above 340 μM) could be associated with bilirubin destructive effects like kernicterus or bilirubin-induced neurological dysfunction. Elevation of serum bilirubin above physiological range leads to yellow coloration of the sclera, mucosal surfaces and skin (Vitek L. & Ostrow J.D., 2009).

Hyperbilirubinemia can be divided into three different categories including pre-hepatic, intra-hepatic, or post-hepatic. Pre-hepatic jaundice is mainly caused by an increased rate of hemolysis. The major cause of enhanced hemolysis is defective plasma membrane of red blood cells. Genetic diseases, such as sickle cell anemia, spherocytosis, thalassemia, pyruvate kinase deficiency, glucose 6-phosphate dehydrogenase deficiency, congenital lecithin-cholesterol acyltransferase (LCAT) deficiency, GSH synthase deficiency and erythroblastosis fetalis can lead to increased red cell lysis and cause hemolytic/pre-hepatic jaundice. Patients with pre-hepatic hyperbilirubinemia are usually presented with dark yellow-brown colored urine due to increased production of urobilinogen and high unconjugated bilirubin levels in serum. Intra-hepatic or hepatocellular hyperbilirubinemia can be caused by acute or chronic hepatitis, hepatotoxicity, cirrhosis, drug-induced hepatitis

and alcoholic liver disease. Due to hepatocyte necrosis, the ability of the liver to metabolize bilirubin is reduced leading to increase of unconjugated and conjugated bilirubin in serum (Beckingham I.J. & Ryder S.D., 2001). Intrahepatic hyperbilirubinemia is commonly seen in newborn, known as neonatal jaundice, caused by immaturity of bilirubin pathway (Ip S. et al., 2004). Congenital disorders of bilirubin pathway are also causes of hepatocellular hyperbilirubinemia such as Gilbert's syndrome (Muchova L. et al., 2004) or Crigler-Najjar syndrome (Bosma P.J., 2003). Defect in bilirubin transport from hepatocyte lead to increase of conjugated bilirubin that is typical for Dubin-Johnson syndrome or Rottor syndrome (Erlinger S. et al., 2014; van de Steeg E. et al., 2012). Post-hepatic or obstructive hyperbilirubinemia is typically caused by extra-hepatic biliary obstruction (Beckingham I.J. & Ryder S.D., 2001).

1.3.1 Gilbert syndrome

Gilbert syndrome is an autosomal dominant hereditary disease characterized by intermittent mild unconjugated hyperbilirubinemia in the absence of hepatocellular disease or hemolysis. Mild unconjugated hyperbilirubinemia is caused by mutation in UGT1A1 promoter region, enzyme responsible for bilirubin glucuronidation, leading to a decreased expression of the enzyme with normal structure and specific activity (approx. 30%). As a result, serum unconjugated bilirubin tends to be elevated by 2–3-fold with serum concentrations typically about 30 μM in Gilbert subjects (McCarty M.F., 2007; Muchova L. et al., 2004). Even though bilirubin and its elevation has been considered a sign of liver failure and potential defect in organism, mild elevation of bilirubin especially seen in Gilbert syndrome patient is associated with protective properties. Several studies have shown that serum bilirubin protects against cardiovascular diseases, peripheral vascular disease and there is an evidence that it may be protective against certain types of cancer (Jiraskova A. et al., 2012; Novotny L. & Vitek L., 2003; Vitek L. et al., 2002; Vitek L. et al., 2006).

1.4 Pharmacological and clinical aspects of heme oxygenase

Role of HMOX respectively its products CO and bilirubin/biliverdin in health and disease is undisputable. HMOX system have proven its role as a beneficial and/or therapeutic

effector in a large number of pathologic conditions including but not limited to diabetes (Tiwari S. & Ndisang J.F., 2014), inflammation (Chung S.W. et al., 2008), heart disease (Akamatsu Y. et al., 2004), hypertension (Motterlini R. et al., 1998), transplantation (Ozaki K.S. et al., 2012), pulmonary disease (Ryter S.W. et al., 2007), obesity (Li M. et al., 2008) and many others. Pharmacological approach in HMOX induction or in some cases inhibition is now under the investigation. The list of compounds able to induce HMOX1 expression is ever-increasing, some of which have therapeutic properties. Statins, competitive inhibitors of 3-hydroxy-3-methylglutaryl co-enzyme A reductase, a rate-limiting enzyme of cholesterol biosynthesis, have shown in several *in vitro* and *in vivo* studies that are capable of HMOX1 induction (Grosser N. et al., 2004; Hsu M. et al., 2006; Muchova L. et al., 2007). But further human studies with clinically relevant doses are required to confirm this phenomenon. Nitric oxide and nitric oxide–releasing compounds also induce HMOX1 via NO–sGC–cGMP pathway. Activation of sGC and increase in cGMP production is probably major pathway leading to HMOX1 induction by NO and NO-releasing compounds (Motterlini R. et al., 1996; Ryter Stefan W. et al., 2006). Natural polyphenols are inducers of HMOX1 by either activation of Kelch-like ECH-associated protein 1 (Keap-1) or direct interaction with Nrf2 (Li C. et al., 2007). Among such inductors we could find curcumin (Motterlini R. et al., 2000), caffeic acid phenethyl ester (Scapagnini G. et al., 2002), resveratrol (Chen C.Y. et al., 2005) and flavonoids (Szabo M.E. et al., 2004).

Nevertheless, in some cases like in for example cancer treatment, rather inhibition than induction is desirable (Halina W. et al., 2010). Inhibition of HMOX1 expression can be achieved either by genetic manipulation using RNAi or CRIPSPR/Cas9 system or using pharmacologic approach. Results using *in vitro* and *in vivo* application of RNAi either as a siRNA (Berberat P.O. et al., 2005) (short interfering RNA) or shRNA (Na H.K. & Surh Y.J., 2014) (short harpin RNA) shows that this possibility is here, but therapeutic application is far from practical use. Inhibition of HMOX1 activity by pharmacological agents is another possibility. Metalloporphyrins such as zinc, tin and chromium protoporphyrins have been used as a major pharmacologic tool for decreasing HMOX1 activity and generated large amount of data clarifying the role of HMOX in physiology (Johnson R.A. et al., 1997; Lyall F. et al., 2000; Tulis D.A. et al., 2001; Vreman H.J. et al., 1993). Unfortunately, experimental and therapeutical application of metalloporphyrins is highly limited. Among a long list of side effects like non selectivity towards HMOX isoforms, induction of HMOX1 mRNA, modulation of activity of other enzymes (cytochrome P 450, NOS, sGC) there is also

photoreactive and induced phototoxicity of these compounds, leading to tissue and organ damage (Podkalicka P. et al., 2018). As an alternative, non-porphyrin based HMOX inhibitors were developed. These inhibitors are imidazole-dioxolane based. First inhibitor, azalanstat, was described by Denagel *et al.* and since its discovery several other inhibitors have been developed. Main advantage of these imidazole-based compounds is their water solubility and enzymatic isoform specificity. Moreover, they do not affect other enzymes like metalloporphyrin and display lower toxicity. But larger pharmacokinetics and pharmacodynamics studies are required to assess potential application in disease processes (Kinobe R.T. et al., 2008; Kinobe R.T. et al., 2006).

2 AIMS

The aim of this thesis was to evaluate metabolic role of HMOX and its products with focus on liver diseases and oxidative stress. Specifically, our aims were:

1. to clarify role of HMOX induction by heme in prevention of estradiol induced cholestasis model in rats and possible mechanism of action.

2. to evaluate pharmacokinetics of inhaled CO in rats.

3. to verify possible anticholestatic role of CO in the treatment of endotoxin-induced cholestasis

4. to clarify whether CO have antiproliferative effect on pancreatic cancer cells

5. to find natural compound(s) that can affect bilirubin metabolism and increase its intracellular as well as systemic concentrations.

3 METHODS

Following list represents the methods used in the submitted dissertation thesis by author. Detail description and other information about particular method are listed in publications related to this thesis in section “Materials and Methods”.

- CO determination in tissue and cell cultures
- COHb determination
- Cultivation of immortalized cell lines (HepG2, CAPAN-2, Patu 8902, BxPC3) and primary rat hepatocytes
- Cytotoxicity measurement (MTT Cytotoxicity Assays)
- Determination of serum bilirubin concentration (LC/MS/MS)
- Determination of tissue bilirubin concentration (HPLC)
- Determination of UGT1A1 activity (UGT-Glo™ assay)
- Gene expression analysis (RT-qPCR)
- HMOX activity measurement
- In vivo experiments
- Malondialdehyde Determination
- Statistical analysis
- Transfection of primary rat hepatocytes using lipofection
- Western blots

4 RESULTS

The results of this thesis are presented in a form of four original manuscript with HMOX as a main topic. Every publication is separately discussed in context of current literature.

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ŠUK, Jakub, Jana JAŠPROVÁ, David BIEDERMANN, Lucie PETRÁSKOVÁ, Kateřina VALENTOVÁ, Vladimír KŘEN, Lucie MUCHOVÁ a Libor VÍTEK. Isolated Silymarin Flavonoids Increase Systemic and Hepatic Bilirubin Concentrations and Lower Lipoperoxidation in Mice. *Oxidative Medicine and Cellular Longevity*. 2019, **2019**, 1-12

5 DISCUSSION

The research of a presented thesis was focused on evaluation of metabolic role of HMOX and its products.

We were interested in significance of HMOX in pathophysiological process, specifically its role in inflammation, hepatoprotection and proliferation. HMOX pathway respectively its products play important role in maintaining homeostasis of organism, and their modulation shows to be protective in numerous *in vitro* and *in vivo* models of various diseases. Bilirubin, yellow bile pigment, for many decades considered only waste product of pathway and sign of liver disease, is also an important antioxidant with immunosuppressive and cytoprotective properties. Nevertheless, possibilities of bilirubin upregulation for this purpose have not been studied in detail before.

HMOX products CO and biliverdin/bilirubin have exerted their hepatoprotective properties with bilirubin protecting from oxidative stress triggered by bile acids (Muchova L. et al., 2011) and CO affecting bile flow and the expression of hepatic transporters (Suematsu M. et al., 1994; Vanova K. et al., 2014) (see below). Based on these implications, our question was if HMOX and its products could possess protective effects in ethinylestradiol (EE) induced cholestasis. This was described in the study entitled „**Protective effect of heme oxygenase induction in ethinylestradiol-induced cholestasis**“ (Muchova L. et al., 2015). EE induced cholestasis was used as an experimental model of human intrahepatic cholestasis. HMOX1 induction was achieved by application of hemin in the form of methemalbumin to experimental rats to diminish the toxicity of free heme which is considered a strong pro-oxidant with harmful effect on various organ systems.

Induction of HMOX in EE-treated animals have shown to have a clearly anti-cholestatic effect as measured by serum cholestatic markers. Similar results were also observed in the expression of key hepatic sinusoidal (OATPS, sodium/taurocholate cotransporting polypeptide (NTCP)) and canalicular (multidrug resistance-associated protein 2 (Mrp2)) transporters, where their repression caused by EE treatment was reversed by HMOX induction. The only transporter specifically activated by heme was Mrp3. Mrp3 is considered one of the basolateral overflow pumps compensating for impaired canalicular Mrp2 (Cao J. et al., 2002; Geier A. et al., 2007). The HMOX induction by heme and Mrp3 regulation is

mediated via Nrf2 pathway. Nrf2 is transcriptional factor responsible for activation of many antioxidative stress genes (Cuadrado A. et al., 2018). Relationship between Mrp3 and Nrf2 and a possible anti-cholestatic effect of this pathway has been described (Maher J.M. et al., 2007; Okada K. et al., 2009). We observed a significant increase of Mrp3 expression in all heme and/or cholestatic groups with the highest increase when both cholestasis and heme were present. Using primary rat hepatocyte and siRNA silencing method, we confirmed the key role of Nrf2 in heme mediated Mrp3 overexpression. In conclusion we demonstrated that the induction of HMOX1 increases hepatocyte transporter expression, subsequently stimulating bile flow in cholestasis.

HMOX has been shown to have hepatoprotective properties that are mediated particularly through the action of its metabolic products, where CO is one of the most significant. CO has been intensively studied since 1993 when its function as a neurotransmitter was discovered. Most prevalent application route for CO is inhalation. However, the data about CO tissue distribution and half-life within an organism, which are critical for evaluation of its biological functions and/or toxicity, are missing. To the date of publication of our study entitled „**Protective effects of inhaled carbon monoxide in endotoxin-induced cholestasis is dependent on its kinetics**“ (Vanova K. et al., 2014) only scarce data on CO elimination profiles and kinetics were described in mice, porcine and ovine models (Åberg A.-M. et al., 2004; Shimazu T. et al., 2000; Vreman H.J. et al., 2005). We described the kinetic profile of inhaled CO in rats as well as the tissue distribution following inhalation. We found that distribution and elimination of CO are tissue-dependent with half-life independent of the CO concentration. Maximum CO was found in blood immediately after inhalation (111- fold increase) followed by spleen, lung, liver, heart, kidney, brain (4 - fold change) while the mean half-life was the highest in the spleen and lowest in the lung. Common tool for CO determination in blood is carbonylhemoglobin (COHb). However, COHb concentrations can be affected by various factors, such as concentration, duration of exposure, time for transport, oxygen saturation, ventilation rate, and the animal model used (Šuk J. & Muchova L., 2018).

As we show in the study and as title implies, the kinetics and half-life of CO matter. We have found that LPS treatment led to significant downregulation of a mRNA expression of hepatic sinusoidal and canalicular transporters. LPS induced proinflammatory cytokines including TNFα, IL-1β and IL-6, have been characterized as mediators of reduction in bile

flow and organic anion excretion (Geier A. et al., 2007). Interestingly CO pre-treatment upregulated the expression of anti-inflammatory cytokine *IL10* and simultaneously significanty downregulated LPS-induced *TNFα* expression at 1h after CO pre-treatment. These results support previous finding about anti-inflammatory properties of CO (Otterbein L.E. et al., 2000). As a result of *TNFα* repression and *IL-10* upregulation, CO pre-treatment normalized mRNA expression of hepatocyte transporters within 1 h after LPS administration. After 1 h from inhalation, more than half of CO is eliminated from the liver (liver half-life 0,6 h), so we assume that the effect of CO on transporter expression is tightly associated with CO concentration in tissues.

Even though this effect was only transient, it was sufficient to significantly decrease serum bile acid levels 12 h after LPS administration clearly indicating that bile accumulation and secretion in cholestasis is a dynamic process reflecting early events in hepatocyte.

CO among other protective properties shows to be effective in inhibition of proliferation (Otterbein L. E. et al., 2003; Song R. et al., 2002). We have studied antiproliferative properties of CO on a model of pancreatic cancer. Pancreatic cancer, having high mortality and recurrence rates, is a type of cancer where even modern medicine have shown to be only moderately effective (Ko A.H. et al., 2008). Akt pathway seems to play important role in pancreatic carcinogenesis (Parsons C.M. et al., 2010). In the study "**Antiproliferative effects of carbon monoxide on pancreatic cancer**" we investigated if CO delivered as a gas and/or in a form of carbon monoxide-releasing molecule (CORM) has effects on pancreatic cancer cell proliferation (Vitek L. et al., 2014). We discovered that exogenously administered CO in gaseous or CORM form acts as a potent inhibitor of pancreatic carcinogenesis. In athymic mice subcutaneously transplanted with human pancreatic xenografts, CO reduced tumor volume, limited tumor neovascularization and profoundly prolonged survival. The mechanism of this action seems to be inhibition of Akt phosphorylation, which in turn is reflected by decreased neovascularization, as observed in our CORM treated mice. Akt pathway is one of the most commonly dysregulated pathways in all types of cancer, ultimately resulting in increased proliferation and a loss of apoptotic signaling and cell growth (Mundi P.S. et al., 2016). Our data are in line with observations of other groups. Wegiel *et al.* have shown that inhaled CO suppressed the growth of prostate cancer xenografts, which was associated with increased tumor cell apoptosis and reduced tumor vascularization (Wegiel B. et al., 2013). Ferrando *et al.* demonstrated that HMOX1

overexpression in prostate cancer cells potently suppressed angiogenesis (Ferrando M. et al., 2011). Even clinical data that are available show better prognosis for colon cancer patients overexpressing HMOX1 in tumor tissues (Becker J.C. et al., 2007).

Tumors in which HMOX1 was genetically silenced grew slower than did tumors expressing normal levels of the enzyme, and this reduced growth was associated with a reduced microvessel density, consistent with the notion that HMOX1 (and CO) facilitates intratumor and peritumor angiogenesis as shown in studies with small interfering RNA (siRNA) pancreatic cancer cells (Berberat P.O. et al., 2005) and mouse hepatocellular carcinoma cell lines (Sass G. et al., 2008).

Studies investigating the effects of HMOX1 inhibitors on tumor angiogenesis and growth have further confirmed the role of CO overproduction in cancer. But there are reports showing that HMOX1 silencing increased tumor growth, suggesting that the role of HO1 and CO in cancer is not fully understood yet and is highly dependent on tumor type (Gueron G. et al., 2009; Zou C. et al., 2011).

Bilirubin, yellow bile pigment is another important product of HMOX pathway. After decades when it has been considered only a toxic waste product particularly in the context of neonate jaundice, bilirubin has established itself as a powerful antioxidant with immunosuppressive and cytoprotective properties (Gazzin S. et al., 2017; Gazzin S. et al., 2016; Wagner K.H. et al., 2015). Mildly elevated bilirubin levels in serum have been shown to protect organism against oxidative stress-mediated diseases including atherosclerosis and cancer, as well as a number of inflammatory, autoimmune and degenerative diseases. Even single micromolar elevation of systemic concentrations of bilirubin contributes to these beneficial effects (McCarty M.F., 2007). This association is clearly evident in subjects with Gilbert syndrome characterized by mild systemic elevations of unconjugated bilirubin. This led us to the idea to pharmacologically induce mild unconjugated hyperbilirubinemia in order to suppress development of oxidative stress-related diseases. Successful elevation of bilirubin without liver damage using natural polyphenols contained in silymarin was demonstrated in the study entitled "**Isolated Silymarin Flavonoids Increase Systemic and Hepatic Bilirubin Concentrations and Lower Lipoperoxidation in Mice**" (Šuk J. et al., 2019).

Strategy for mild bilirubin elevation can be either increasing its production by induction/upregulation of HMOX1 (Muchova L. et al., 2007; Zelenka J. et al., 2012), or decreasing its elimination by inhibition of UGT1A1 (Dekker D. et al., 2011), the key enzyme responsible for bilirubin conjugation in the liver tissue.

Based on published data, inhibition of UGT1A1 can be feasible using flavonolignans and other natural polyphenols, especially silymarin and/or its individual constituents (Saracino M.R. & Lampe J.W., 2007). Silymarin, an extract from *Silybum marianum* seeds, has been widely used for decades for its hepatoprotective properties. Although silymarin use has been well established, from clinical practice point of view its use is contradictory. *Silybum marianum* seeds consist of approximately 70% to 80% of the silymarin complex and an approximately 20% to 30% chemically undefined fraction, comprising mostly polymeric and oxidized polyphenolic compounds. Major component of silymarin complex is silybin forming approximately 30% of silymarin complex. Besides silybin, which is a mixture of 2 diastereomers in approximately 1:1 proportion, considerable amounts of other flavonolignans are present in the silymarin complex, namely isosilybin, dehydrosilybin, silychristin, and silydianin, and a few flavonoids, mainly taxifolin (Simanek V. et al., 2000). The main reason for controversial outcomes of experimental and/or clinical studies and uncertainty of silymarin effects is a variable composition of silymarin preparations used in most of the studies. It has been well documented that proportions of respective components in various silymarins depend largely on the source of *S. marianum* seeds (plant cultivar and cultivation conditions) and also on the extraction and processing procedures (Kren V. & Walterova D., 2005; Simanek V. et al., 2000). We focused on individual components of silymarin and their biological properties because biological effects of these components might differ considerably. For example silybin and silychristin improve the metabolic activity of kidney cells (proliferation rate, proteosynthesis, and LDH activity), whereas isosilybin and silydianin are inactive in this regard (Sonnenbichler J. et al., 1999). Our *in vitro* results show that flavonolignans of silymarin inhibit *UGT1A1* mRNA expression and also increase intracellular concentration of UCB *in vitro* among those the dehydrosilybin was the most potent flavonolignan.

Silymarin, respectively its main constituent silybin, are well known inhibitors of UGTs activity and mRNA expression (Mohamed M.E. & Frye R.F., 2011; Mohamed M.F. et al., 2010). However, the role of dehydrosilybin on UGT1A1 expression and activity has never been studied. Dehydrosilybin is mainly studied for its radical scavenging, anti-inflammatory

and anti-cancer properties (Karas D. et al., 2016; Pyszkova M. et al., 2016). In our study, we have shown that dehydrosilybin affects bilirubin pathway and is even better inhibitor of UGT1A1 than silybin (dehydrosilybin A 2,1 μM and 4,3 μM silybin).

The main question was, if the intraperitoneal and/or oral treatment with dehydrosilybins can increase intracellular as well as systemic concentrations of UCB *in vivo*. We successfully demonstrated that both application routes led to significant increase in both systemic and intracellular concentrations of UCB. Also serum concentrations of UCB were similar to those observed in mice with Gilbert syndrome genotype (Hinds T.D., Jr. et al., 2016), suggesting possibility that "iatrogenic Gilbert syndrome" can be achieved by dehydrosilybins treatment.

Dehydrosilybins belong to the minor flavonoids of the silymarin complex, usually accounting for 1-2% of all flavonolignans (1.8% in the preparation used in the present work). But their biological effects might be of a real clinical importance. For example, their anti-aggregation and anti-oxidative properties results in lifespan extension of *Caenorhabditis elegans* (Filippopoulou K. et al., 2017). The antioxidant effects of dehydrosilybins was also observed in our study. Decreased malondialdehyde concentrations in the livers of mice treated with dehydrosilybins might have been mediated, at least partially, via increased intracellular bilirubin concentrations.

Bilirubin and its modulation by silymarin components has not been a primary objective of clinical studies. Since silymarin and its constituents are mainly studied in liver diseases associated with rapid elevation of bilirubin due to liver damage, decrease of bilirubin concentration is rather desirable in these situations (Saller R. et al., 2008). It has been shown that silymarin constituents have ability to scavenge free radicals and increase cellular GSH content, prevent cirrhosis by inhibition of myofibroblast formation, to enhance hepatocyte regeneration by proteosynthesis stimulation, to regulate membrane permeability and increase its stability and to have immunomodulatory effects on the liver tissue(Gazak R. et al., 2007; Morazzoni P. & Bombardelli E., 1995). Modulation of bilirubin pathway could be also one of the hepatoprotective mechanism of silymarin.

In conclusion, our data demonstrate possibility of modulation of bilirubin pathway by natural polyphenols contained in silymarin. Elevation of serum UCB by natural polyphenols could be a safe way for protection against oxidative stress related diseases including

atherosclerosis, cancer or diabetes. This mechanism might also contribute to the hepatoprotective mechanism of silymarin.

6 SUMMARY

Components of HMOX pathway like heme, CO and bilirubin have been for a long time considered only toxic waste products. But recent findings well documented their beneficial and signaling properties. Heme is an important signaling molecule, CO displays, among others, anti-inflammatory, anti-apoptotic, anti-proliferative and anti-coagulative effect and bilirubin is a powerful endogenous antioxidant with immunomodulatory, anti-inflammatory and antiproliferative properties.

In the presented thesis, we investigated the metabolic role of HMOX and its products. In detail, the role of HMOX induction by heme, pharmacological properties of inhaled CO and its role in cholestasis and cancer and possibility of bilirubin serum elevation by natural polyphenols were investigated.

HMOX has shown its indispensable role in defense against oxidative stress-mediated diseases but its role in cholestasis hasn´t been studied yet. We demonstrated that the induction of HMOX1 increases hepatocyte transporter expression, subsequently stimulating bile flow in cholestasis.

CO is also an important signaling molecule with wide therapeutic potential. However, information about pharmacokinetics in different animal models were missing. We found that distribution and elimination of CO in rats is tissue-dependent with its half-life independent of the CO concentration. We also showed that CO exposure substantially attenuated endotoxin-induced cholestatic liver injury, an effect directly related to the kinetics of inhaled CO.

One of the area, where the role CO is not fully understood, is cancer. We found that CO in relatively low doses has an antiproliferative effect on pancreatic cancer cell lines and acts as a potent antiproliferative agent.

The increase of systemic levels of unconjugated bilirubin could be one of the solutions for preventing oxidative stress related diseases. We identified natural polyphenols contained in milk thistle that affect hepatic and serum bilirubin concentrations, as well as lipoperoxidation in the liver.

To conclude, we presented the evidence of an importance of HMOX and its metabolic products in several crucial steps of cellular pathways and homeostasis. Moreover, we presented that modulation of HMOX pathway might represent a potential therapeutic strategy for the treatment of various disorders associated with oxidative stress and inflammation including but not limited to cholestasis, metabolic and cardiovascular disorders or cancer.

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8 LIST OF ABBREVIATIONS

ABC - ATP- binding cassette

Ahr - arylhydrocarbon receptor

Akt – protein kinase B

ARE – antioxidant response element

ARNT - AhR nuclear translocator

BV – Biliverdin

BVR – biliverdin reductase

CB - conjugated bilirubin

Cas9 - CRISPR associated protein 9

cGMP – cyclic guanosine monophosphate

cJNK - Jun N-terminal kinases

CO – carbon monoxide

COHb – carbonyl hemoglobin

CORM - carbon monoxide releasing molecule

CRIPSPR - Clustered Regularly Interspaced Short Palindromic Repeats

CREB - cAMP response element-binding protein

DRE - drug responsive element

EE – Ethinylestradiol

EGR1 - Early growth response protein 1

ERG1 - ETS domain- containing protein

ERK - extracellular signal-regulated kinase

GTP - guanosine triphosphate

HDL - high density lipoprotein

HIF1α - hypoxia inducible factor

HMOX - Heme oxygenase

HRE – hormone response element

HSF1 – heat shock factor 1

HSP 70 – heat shock protein 70

I/R – ischemia reperfution

IL-1β – interleukin 1 β

IL-6 – interleukin 6

IL-10 – interleukin 10

Keap-1 - Kelch-like ECH-associated protein 1

LC/MS/MS – Liquid chromatography tandem mass spectrometry

LCAT – Lecithin–cholesterol acyltransferase

LPS - Lipopolysaccharide

MAPK - mitogen-activated protein kinase

MARE - Maf antioxidant responsive elements

MRP – Multidrug resistance-associated protein

MTT - 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

NADPH - Nicotinamide adenine dinucleotide phosphate

NF-κB - nuclear factor kappa-light-chain-enhancer of activated B cells

NOS – nitric oxide synthase

Nrf2 - nuclear factor (erythroid-derived2)-like2

NTCP – sodium/taurocholate cotransporting polypeptide

OATP - organic anion transporting polypeptides

PI3K – phosphatidyl inositol (3,4,5) - trisphosphate

PKC - proteinkinase C

PPAR-γ - peroxisome proliferator-activated receptor

RNAi - RNA interference

RXR - pregnance X receptor

sGC - soluble guanylate cyclase

shRNA - short harpin RNA

siRNA - short interfering RNA

TGF-β - Transforming growth factor β

TNFα – tumor necrosis factor α

TLR4 - toll-like receptor 4

UCB – unconjugated bilirubin

UGT1A1 - UDP- glucuronosyl transferase 1A1 isoform

VCAM- 1 - Vascular cell adhesion protein 1

XRE - xenobiotic responsive element

9 ANNEXES

Annex 1

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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

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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.

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*.

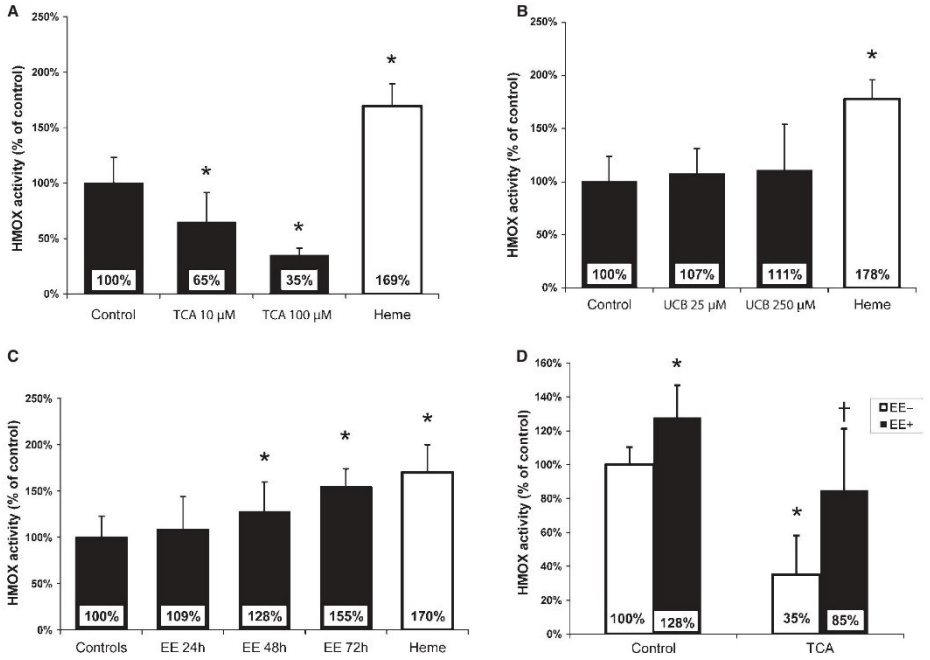

Fig. 1 Total heme oxygenase (HMOX) activity in primary rat hepatocytes. Primary hepatocytes were incubated with: (**A**) taurocholic acid (TCA), (**B**) unconjugated bilirubin (UCB) for 24 hrs or with (**C**) 10 μM ethinylestradiol (EE) for 24, 48 and 72 hrs. (**D**) 10 μM EE was added to media 24 hrs 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

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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*.

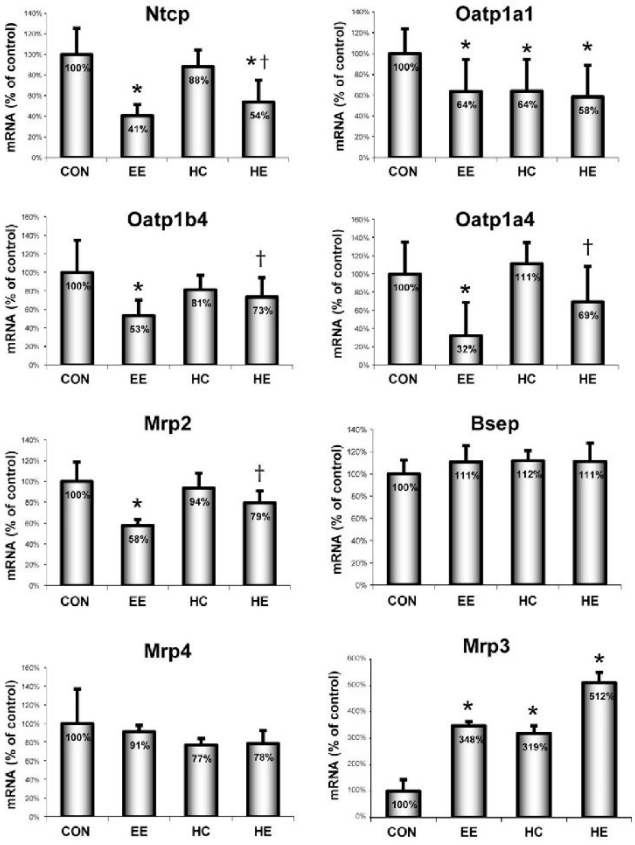

Fig. 2 mRNA expression of key hepatic transporters. Relative expression of key sinusoidal (*Ntcp, Oatps, Mrp3* and *Mrp4)* and canalicular (*Mrp2, Bsep*) bile acid (BA) transporters was measured in the livers of control (CON), ethinylestradiol (EE), heme (HC) or heme + EE (HE)-treated animals. *$P \leq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

(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.

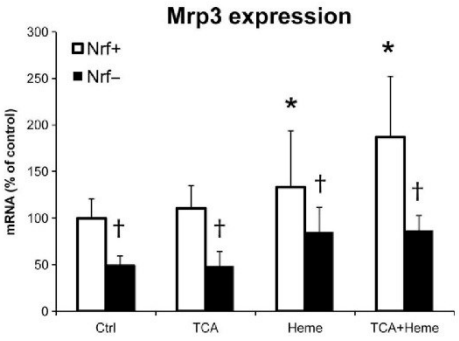

Fig. 3 Mrp3 expression in primary rat hepatocytes. Relative expression of *Mrp3* transporter mRNA was measured in primary hepatocytes with (Nrf−) or without (Nrf+) Nrf2 silencing. Cells were treated with 50 μM taurocholic acid (TCA), 30 μM heme or both for 4 hrs. $*P \leq 0.05$ *versus CON*, $^{\dagger}P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

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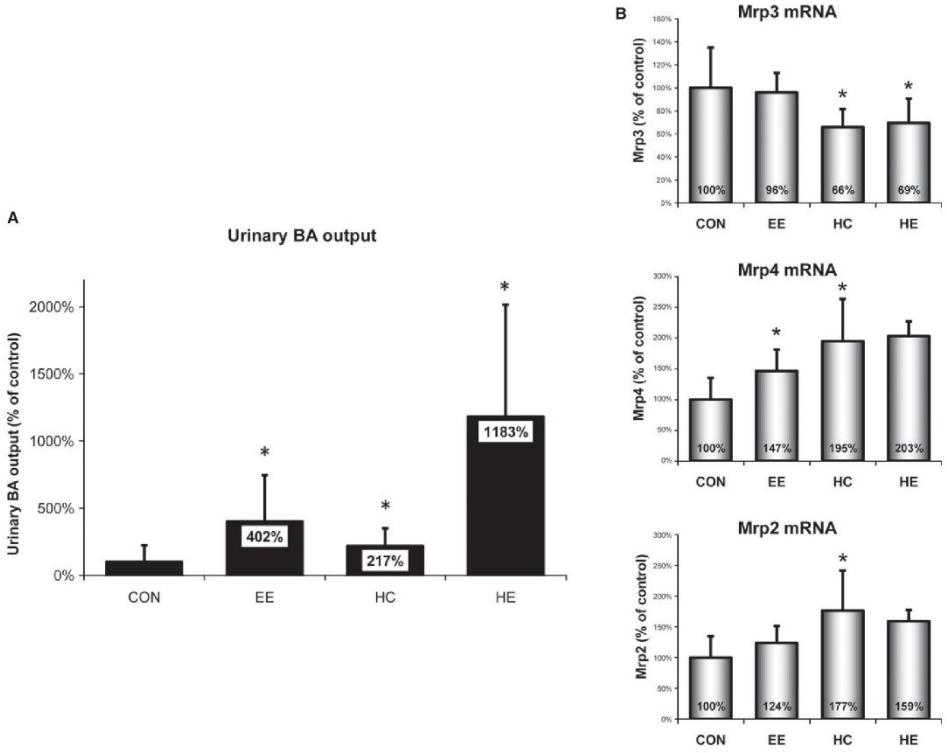

Fig. 4 Urinary bile acid (BA) output and mRNA expression of renal BA transporters. (**A**) BA concentration was measured in the urine collected for 30 min. from control (CON), ethinylestradiol (EE), heme (HC), or heme + EE (HE)-treated animals and expressed as relative changes from CON. (**B**) Relative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

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

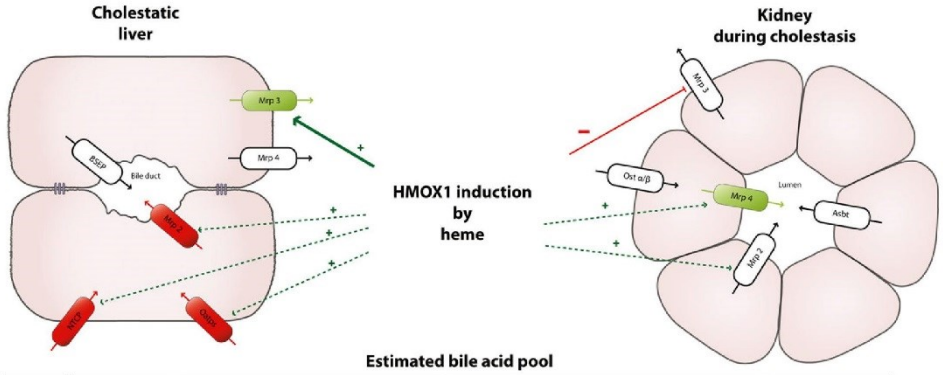

Fig. 5 Proposed mechanism of heme oxygenase-1 (HMOX1) induction on liver and kidney transporters in EE-induced cholestasis. EE-induced cholestasis is characterized by either decrease (red ovals) or increase (green ovals) in the expression of key hepatocyte and kidney transporters. Induction of HMOX1 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.

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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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Annex 2

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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

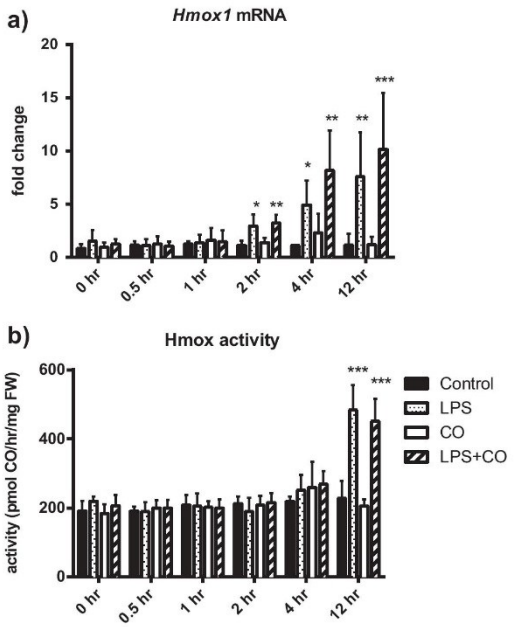

Fig. 1. The effect of LPS and CO on a) *Hmox1* mRNA expression and b) Hmox activity in the liver. a) *Hmox1* mRNA was significantly up-regulated 2 h after LPS administration in LPS- and LPS + CO-treated groups and gradually increased over time. b) Hmox activity significantly increased after 12 h in both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

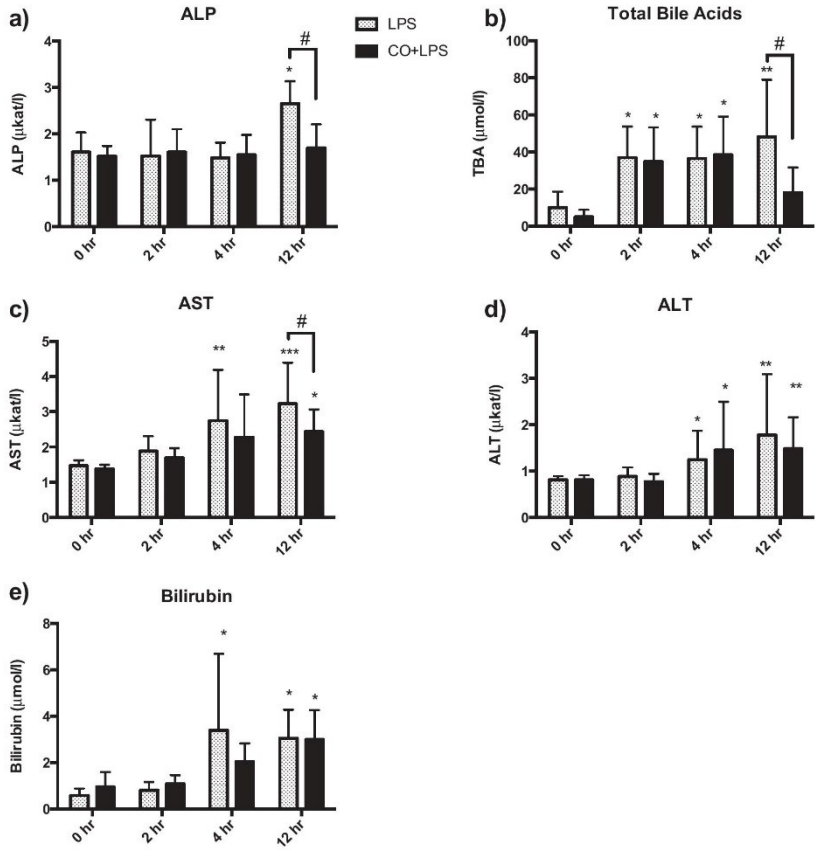

Fig. 2. The effect of LPS and CO exposure on markers of liver injury. Total serum bile acids (TBA), AST, ALT were elevated 4 h after LPS administration. CO pre-treatment decreased the elevation of: a) ALP; b) TBA; and c) AST 12 h after LPS administration. d) ALT activities were significantly elevated 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.

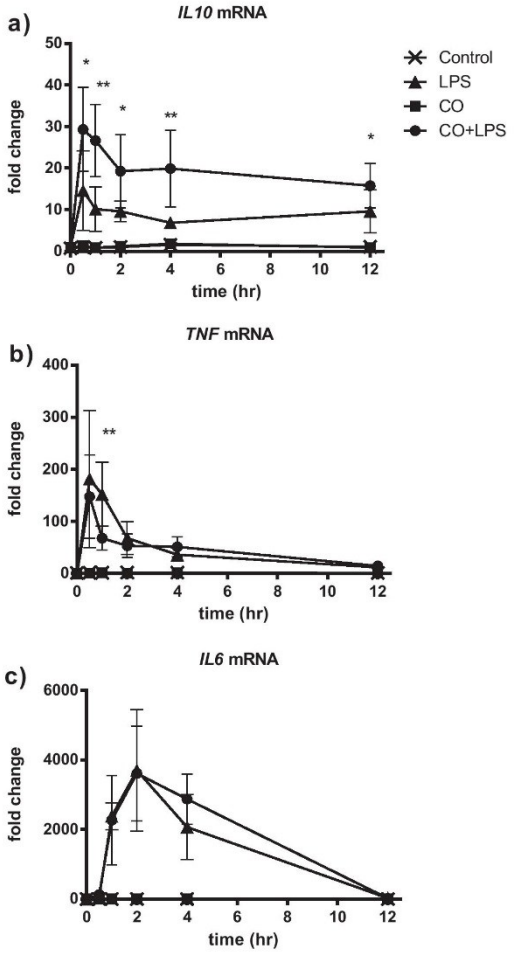

Fig. 3. The effect of CO on expression of inflammatory cytokines in the liver. The expression of cytokines: a) *IL10*; b) *TNF*; and c) *IL6* were measured in hepatic tissue over time. a) Anti-inflammatory *IL10* mRNA was elevated within 0.5 h from LPS treatment at all time points, and significantly 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

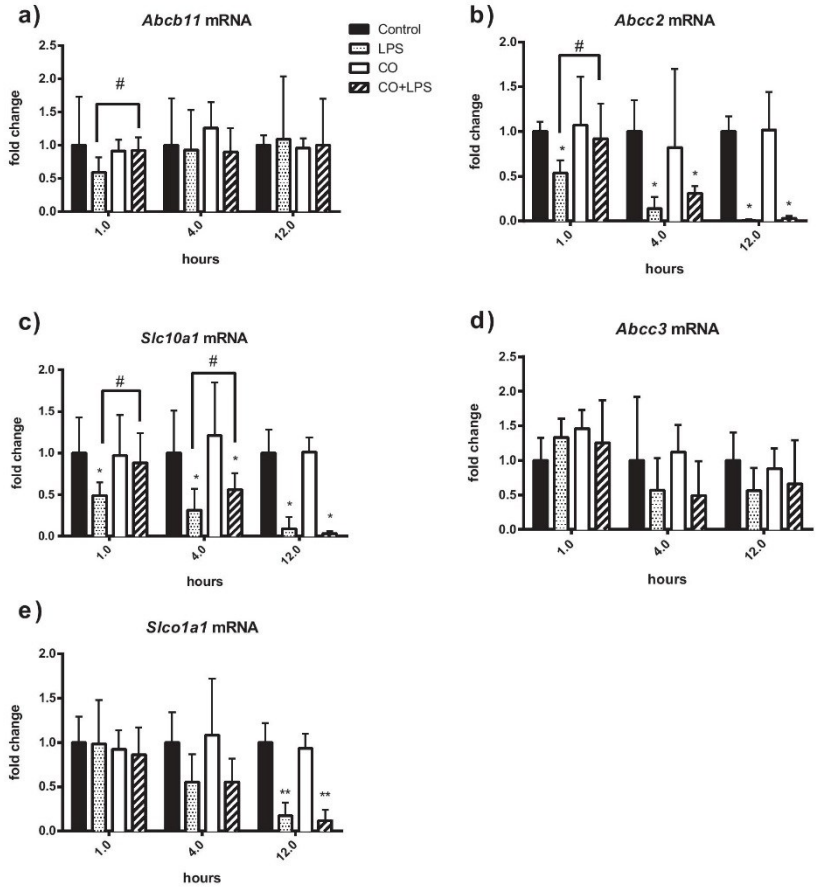

Fig. 4. Effect of CO treatment on expression of hepatic transporters. The expression of hepatic transporter mRNA was measured at different time points after LPS administration. a) *Abcb11* down-regulation was prevented by CO treatment 1 h after LPS; b) *Abcc2* down-regulation induced by LPS was suppressed 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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Annex 3

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Oncology

Antiproliferative effects of carbon monoxide on pancreatic cancer

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ABSTRACT

Background: Carbon monoxide, the gaseous product of heme oxygenase, is a signalling molecule with a broad spectrum of biological activities. The aim of this study was to investigate the effects of carbon monoxide on proliferation of human pancreatic cancer.
Methods: *In vitro* studies were performed on human pancreatic cancer cells (CAPAN-2, BxPc3, and PaTu-8902) treated with a carbon monoxide-releasing molecule or its inactive counterpart, or exposed to carbon monoxide gas (500 ppm/24 h). For *in vivo* studies, pancreatic cancer cells (CAPAN-2/PaTu-8902) were xenotransplanted subcutaneously into athymic mice, subsequently treated with carbon monoxide-releasing molecule (35 mg/kg b.w. i.p./day), or exposed to safe doses of carbon monoxide (500 ppm 1 h/day; $n = 6$ in each group).
Results: Both carbon monoxide-releasing molecule and carbon monoxide exposure significantly inhibited proliferation of human pancreatic cancer cells ($p < 0.05$). A substantial decrease in Akt phosphorylation was observed in carbon monoxide-releasing molecule compared with inactive carbon monoxide-releasing molecule treated cancer cells (by 30–50%, $p < 0.05$). Simultaneously, carbon monoxide-releasing molecule and carbon monoxide exposure inhibited tumour proliferation and microvascular density of xenotransplanted tumours ($p < 0.01$), and doubled the survival rates ($p < 0.005$). Exposure of mice to carbon monoxide led to an almost 3-fold increase in carbon monoxide content in tumour tissues ($p = 0.006$).
Conclusion: These data suggest a new biological function for carbon monoxide in carcinogenesis, and point to the potential chemotherapeutic/chemoadjuvant use of carbon monoxide in pancreatic cancer.

1. Introduction

For decades, carbon monoxide (CO) exposure has been considered a potential threat to human health, and the endogenous production of this gaseous molecule was only thought to be a waste product, a biologically inactive by-product of heme catabolism. However, it is now widely accepted that CO formed endogenously by heme oxygenase (HMOX) confers cytoprotection against tissue and cellular injury [1,2]. CO acts as a smooth muscle relaxant as well as an inhibitor of platelet aggregation *via* guanylate cyclase and cGMP generation [2]. The functional properties of CO have often been compared with nitric oxide (NO), another endogenous gaseous molecule. Indeed, CO shares a number of biological functions analogous to NO [1]. The ability of both NO and CO to act as a vasodilator and to modulate endothelial cell permeability makes it plausible that they could also play essential roles in angiogenesis. It has previously been reported that NO has a dual effect on angiogenesis, and can either promote or inhibit angiogenesis in a dose-dependent manner [3]. Although certain studies have reported that CO is pro-angiogenic [4], the similarity of CO to NO could lead to the speculation that CO might also inhibit carcinogenesis, at least partially, *via* the suppression of angiogenesis. Under stress conditions, the production of CO is increased owing to the upregulation of the stress-responsive heme oxygenase isoenzyme,

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HMOX1 (OMIM*141250) [5]. The recent discovery of transition metal carbonyls that act as CO-releasing molecules (CORMs) has provided a new impetus for the investigation of CO as a cellular messenger, as well as a potential therapeutic agent [6,7]. Both CORMs and CO at doses free from toxic side-effects have been shown to exert important biological functions in numerous model *in vitro* and *in vivo* systems, including vasodilating, antiproliferative, anti-inflammatory effects, contributing to the amelioration of many pathological conditions such as ischaemia – reperfusion injury, inflammatory bowel disease, and organ rejection (for a comprehensive review of the biological effects of CO, see Motterlini and Otterbein [7]). A wide range of CORMs/CO dosages have been tested in these models, depending on CORM type, the means of delivery, and the model used [7]. Significantly, the CO delivered was demonstrated to be non-toxic for healthy tissues, when keeping the CO haemoglobin levels within safe levels [8]. Based on these data, a CO inhalation system for human use has been developed and used in the first clinical trials [7].

Pancreatic tumours, having high mortality and recurrence rates, are an example of a tumour type in which any type of medical therapy has, at best, been only modestly effective [9]. Thus, the effective therapy for pancreatic cancer depends on the search for alternative therapeutic modalities that have the potential to inhibit multiple signalling pathways. Although pancreatic carcinogenesis is a very complex issue, with numerous intracellular pathways involved, the phosphatidylinositol-3 kinase/Akt (protein kinase B) seems to play a key role [10]. Akt activation is frequent in pancreatic cancer and correlates well with prognosis [11]; its inhibition has been reported to sensitise cancer cells to the tumour-suppressive effects of chemotherapy [12,13].

All of these facts led us to investigate the potential antiproliferative effects of CO and/or CORM-2 (a ruthenium-based, lipid-soluble CORM) on human pancreatic cancer, using experimental *in vitro* and *in vivo* models, with a special focus on the possible CO-mediated effects on Akt phosphorylation.

2. Methods

2.1. Reagents

All cell culture reagents and chemicals, and tricarbonyldichlororuthenium(II) dimer ($[Ru(CO)_3Cl_2]_2$) (more commonly known as CORM-2), were obtained from Sigma Aldrich (Prague, Czech Republic). The CO (500 ppm) gas mixture (20% O_2, 0.03% CO_2, remainder nitrogen) for *in vitro* studies was obtained from Linde Gas (Prague, Czech Republic).

2.2. Cell cultures

The pancreatic cancer cell lines CAPAN-2, BxPc3 (ATCC, Manassas, VA, USA), and PaTu-8902 (DSMZ, Braunschweig, Germany) were used for the *in vitro* studies. The cell lines were cultured as described previously [14]. The cell suspensions ($2 \times 10^{5-6}$ cells/ml) were used for the inoculation of individual wells in the 6-well plate. Cells were treated with either a ruthenium-based CORM (CORM-2, 50 μmol/L; this relatively high concentration was used for all *in vitro* studies because of the very short half-life of CORM-2 [7]) or its inactive counterpart, iCORM-2 (a CO-free CORM-2). The use of iCORM-2 as a control is important, since the Ru-based carrier molecule may exert some biological properties itself. Either the CORM-2 or iCORM-2 was dissolved in a solution of DMSO in PBS (final concentration of DMSO did not exceed 1%, vol/vol), on a daily basis for a period of three days. The plates were incubated at 37 °C in a humidified atmosphere of 5% CO_2 in air. The iCORM-2 was prepared by leaving CORM-2 at room temperature for 2 days, and flushing with nitrogen to remove the residual CO [6]. Before initiating the experiments, the release of CO from freshly diluted CORM-2 was confirmed by gas chromatography (for methodology see below). Alternatively, pancreatic cancer cells, cultured in an analogous manner to that described above, were directly exposed to CO (500 ppm) for 24 h using specific air jars (Oxoid CZ, Thermo Fisher Scientific, Prague, Czech Republic). After treatment, the cells (experiments were performed in triplicate) were washed with PBS, harvested by 0.25% trypsin, and re-suspended. Both cell growth and viability were assessed by the direct counting of trypan blue dye (0.4%) excluding cells.

To study the distribution of CO within the cells exposed to CORM, CORM-2 was incubated with PaTu-8902 pancreatic cancer cells for 75 min at 37 °C in a humidified atmosphere of 5% CO_2 in air. The iCORM-2 and 1% DMSO in PBS were used as controls. The CO concentration in the cells and media were measured as described below.

2.3. Determination of Akt phosphorylation

Akt phosphorylation in cancer cell lysates was determined by ELISA (based on anti-phospho-Akt Ser473 antibody, SuperArray Bioscience Corporation, MD, USA), after treatment of CAPAN-2 pancreatic cancer cells with CORM-2/iCORM-2 (50 mol/L) for 75 min, according to the manufacturer's instructions. Cells exposed to 1% DMSO in PBS (solvent for CORM-2) were also compared with untreated cells. Experiments were performed in hexaplets.

Simultaneously, Western blot analyses of phosphorylated Akt protein were performed on the CAPAN-2 and PaTu-8902 pancreatic cancer cells, treated in an identical manner (CORM-2/iCORM-2, 50 mol/L, 75 min incubation). The cells were lysed in Cell Lysis Buffer (Cell Signaling Technology, MA, USA), according to the manufacturer's instructions. Protein concentration was determined using a BCA assay (Thermo Scientific, IL, USA). Thirty micrograms of protein lysate were separated by SDS-PAGE electrophoresis (10% gel). The proteins were transferred to a PVDF membrane and then immunoblotted with anti-phospho-Akt (Ser473), anti-Akt, and anti-β-actin (Cell Signaling Technology, MA, USA). Antibodies were detected using Goat Anti-Rabbit IgG H&L (HRP) antibody (Abcam, UK), and analysed by ECL (LumiGLO®, Cell Signaling Technology, MA, USA). A Fusion Fx7 device and Bio-1D software (Vilber Lourmat, France) were used to quantify the signals. Results were expressed as the percentage of the total Akt level that was phospho-Akt.

2.4. In vivo tumour models

Six- to eight-week-old athymic mice (strain CD-1, Charles River WIGA, Sulzfeld, Germany) were transplanted subcutaneously with either 10^7 human CAPAN-2 or PaTu-8902 pancreatic cancer cells mixed with matrigel. Seven to ten days after tumour cell implantation, the CAPAN-2-bearing mice received a daily intra-peritoneal treatment of either CORM-2 or iCORM-2 ($n = 6$ in each group) dissolved in 1% DMSO in PBS (35 mg/kg), whereas the PaTu-8902-bearing mice were exposed for 1 h daily to either 500 ppm CO in the synthetic air or ambient air *per se*. The primary outcome of this type of *in vivo* study was the survival time; tumour progression was assessed simultaneously as well (tumour size was monitored every three days in all groups, and the tumour volume determined as described previously [15]). In an additional *in vivo* study, the animals treated with CORM-2 or iCORM-2 ($n = 6$ in each group) were sacrificed at day 14 for the quantification of the capillary density of the tumour. Finally, other sets of animals ($n = 6$ for each group) were exposed to CO (500 ppm of CO in synthetic air for 1 h a day). These animals were either sacrificed immediately after the last CO exposure, for the determination of COHb and CO content in the tumour

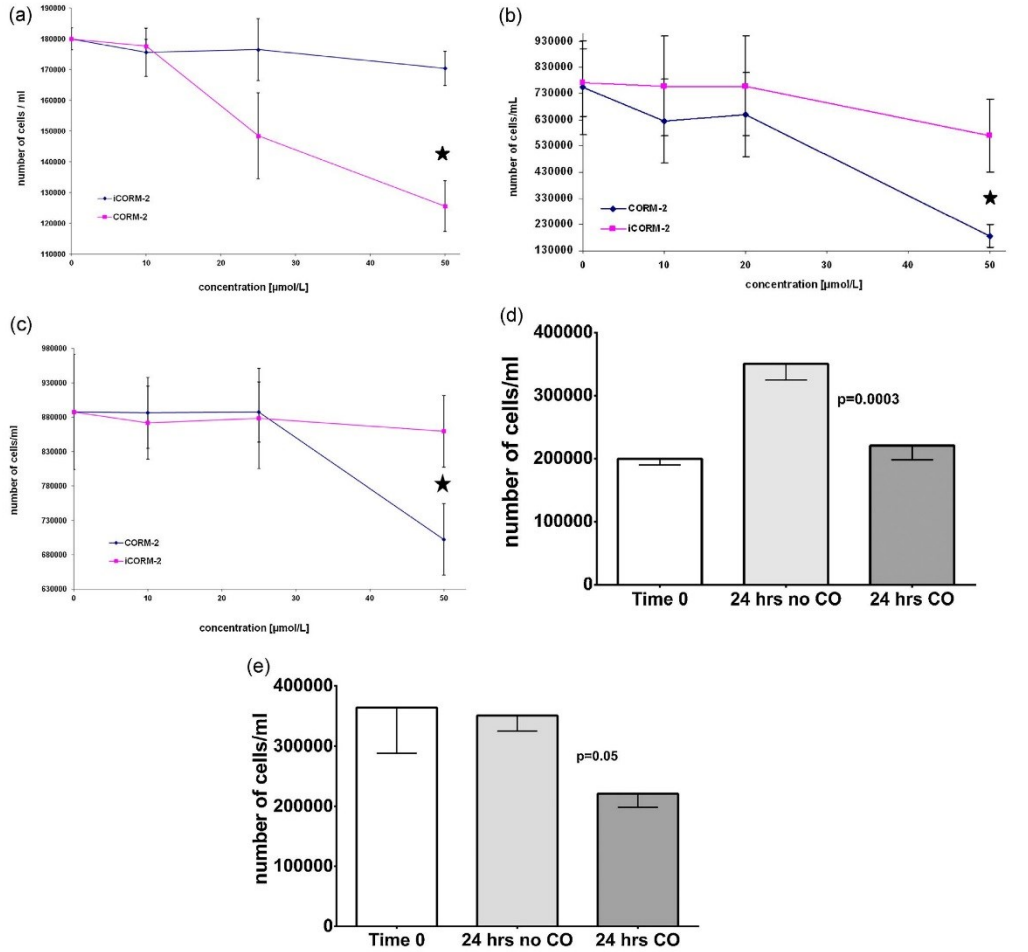

Fig. 1. CO inhibits pancreatic cancer cell proliferation *in vitro*. The effect of CORM-2 (48-h exposure) on pancreatic cancer cell lines: (a) PaTu-8902; (b) CAPAN-2; (c) BxPc3. $^{*}p < 0.05$ (CORM-2 treatment *vs.* iCORM-2, based on ANOVA on Ranks with Dunn's *post hoc* testing). ANOVA, analysis of variance; CORM, carbon monoxide-releasing molecule; iCORM, inactive CORM. The effect of 24-h CO exposure (500 ppm) on the proliferation of human pancreatic cancer cell lines: (d) PaTu-8902; (e) CAPAN-2; $n = 6$ for each group. CO, carbon monoxide.

tissue; or with an 8-h latency after the last exposure, to assess the long-term distribution of CO in various organs (liver, heart, spleen, kidneys, lung and brain).

The local animal research committee approved the protocols for all aspects of the animal studies in accordance with the Guide for the Care and Use of Laboratory Animals, as adopted and promulgated by the United States National Institutes of Health.

2.5. CO measurement

For the determination of CO in the tissues, samples (150–200 mg) were harvested, thoroughly washed in heparinized saline, diluted 1:4 (w/w) in ice-cold reaction buffer (0.1 M PBS, pH 7.4), diced, and sonicated with an ultrasonic tissue disruptor (model XL2000, Misonics, Framingdale, NY, USA). Forty microliters of tissue sonicate were added to CO-free, septum-sealed vials containing 5 μL of 30% (w/v) sulphosalicylic acid. After 30 min incubation on ice, the CO released into the vial headspace was quantified by gas chromatography with a reduction gas analyser (Trace Analytical, Menlo Park, CA, USA), as previously described [16]. This method has a detection limit of 1 pmol of CO, with a linear range of 1–500 pmol of CO. Tissue CO content was calculated as pmol of CO per mg of tissue. CO content in the cells (expressed as pmol of CO per mg of protein) and in the media from the *in vitro* experiments were measured in an analogous way.

To determine CO liberation from CORM-2, CORM-2 or its inactive counterpart (iCORM2) were dissolved in 1% DMSO in PBS and immediately mixed with the mouse blood in a CO-free vial. Next,

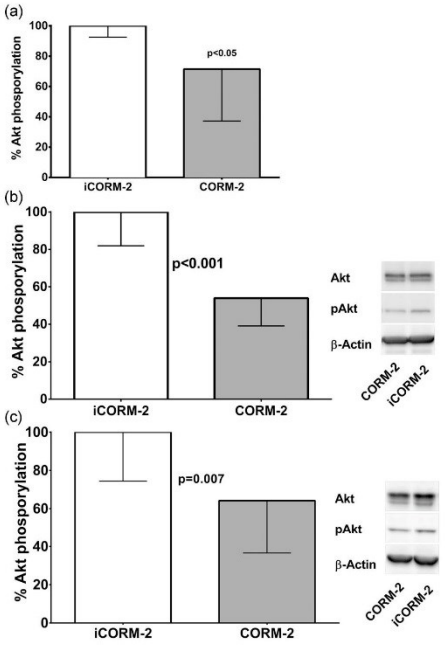

Fig. 2. CORM-2 inhibits Akt protein phosphorylation in pancreatic cancer cells. (a) Akt protein phosphorylation in CAPAN-2 cell lysates determined by ELISA. (b) Akt protein phosphorylation in CAPAN-2 cell lysates determined by Western blot. (c) Akt protein phosphorylation in PaTu-8902 cell lysates determined by Western blot. CAPAN-2 and PaTu-8902 pancreatic cancer cells were exposed to CORM-2 (50 μmol/L, 75 min exposure); data expressed as mean ± SD; $n = 6$ for each group. Two bands of Akt protein correspond to different Akt isoforms [36]. CORM, carbon monoxide-releasing molecule; iCORM, inactive CORM.

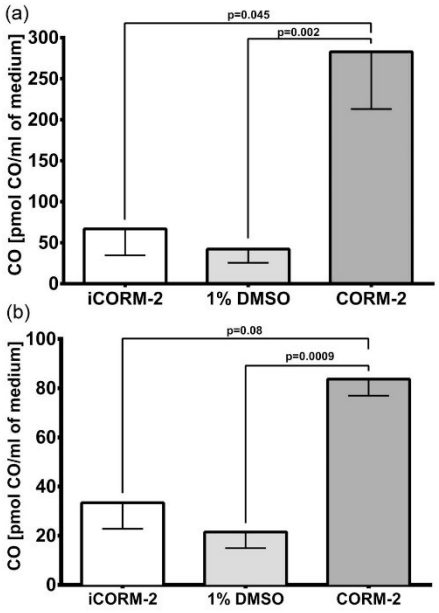

Fig. 3. CO distribution in the PaTu-8902 pancreatic cancer cells exposed to CORM-2. (a) CO content within PaTu-8902 pancreatic cancer cells exposed to CORM-2 (50 mol/L, 75-min exposure). (b) CO content in the culture media of PaTu-8902 pancreatic cancer cells exposed to CORM-2 (50 μmol/L, 75-min exposure); $n = 6$ for each group. CO, carbon monoxide; CORM, carbon monoxide-releasing molecule; iCORM, inactive CORM.

CO-haemoglobin formation was measured by gas chromatography as previously described [17]. The CO-haemoglobin method has a detection limit of 1 pmol of CO per vial.

2.6. Immunohistochemistry and quantification of capillary density

The immunohistochemistry was performed as previously described [18] on frozen or formalin-fixed, paraffin-embedded, randomly sampled, 3-μm sections of tumours with a 1:100 dilution of rat anti-mouse CD31 (BD Biosciences Oxford, UK), peroxidise-labelled rabbit anti-rat antibody (1:250). The mean density of CD31-positive vessels was determined in two areas per section per animal, with these images captured and analysed using Image Pro Plus image analysis software (Media Cybernetics Europe, Berkshire, UK).

2.7. Statistical analyses

All data are presented as the mean ± SD, or median and 25–75% range, when the data were non-normally distributed. Statistical comparisons of *in vitro* data were performed using ANOVA followed by the Student–Newman–Keuls test, or ANOVA on ranks with Dunn's *post hoc* testing, as appropriate. The statistical significance of differences between *in vivo* variables was evaluated by t-test or the Mann–Whitney rank sum test. Kaplan–Meier log rank survival analysis with Holm–Sidak *post hoc* testing was used to assess the effect of CORM-2 treatment on the survival of the animals. Group mean differences in tumour size were measured by repeated measures analysis of variance (RM ANOVA) with Holm–Sidak *post hoc* testing. When needed, log transform values of tumour size were used for comparisons to comply with equal variance requirements. Statistical significance was set at the value of $p < 0.05$.

3. Results

3.1. CO inhibits pancreatic cancer cell proliferation in vitro

Carbon monoxide, in the form of CORM-2, markedly inhibited proliferation of all tested human pancreatic cancer cell lines in a dose-dependent manner ($p < 0.05$, Fig. 1a–c).

To analyse whether CO in the form of CORM-2 might affect phosphorylation of the Akt protein (one of the key events in pancreatic carcinogenesis [10–13]), CAPAN-2 pancreatic cancer cells were treated with CORM-2 (50 μmol/L). Consistent with its antiproliferative effects, this concentration of CORM-2 was found to inhibit Akt phosphorylation in CAPAN-2 and PaTu-8902 pancreatic cancer cells by 30–50% compared with iCORM-2 (its inactive counterpart), as confirmed by both ELISA and Western blotting analyses ($p < 0.05$, Fig. 2a–c).

In order to assess whether the observed antiproliferative effects were only related to CO released from the CORM-2 molecule, PaTu-8902 and CAPAN-2 pancreatic cancer cells were directly exposed to CO in synthetic air (500 ppm) for 24 h. In these studies, CO clearly prevented proliferation of the PaTu-8902 cells ($p < 0.05$, Fig. 1d),

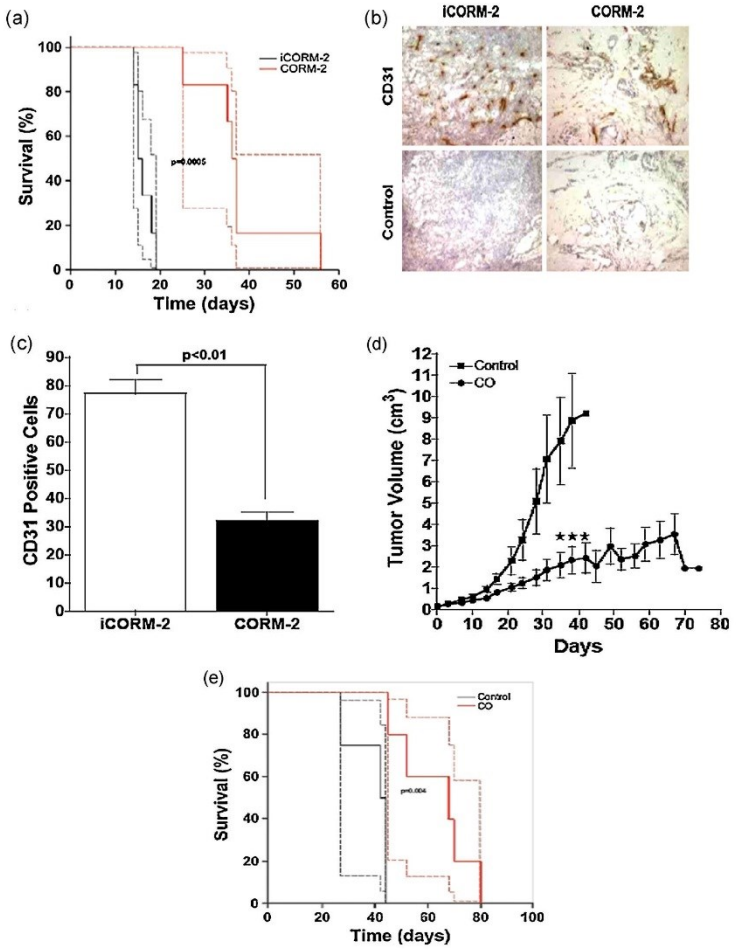

Fig. 4. CO inhibits pancreatic tumour progression *in vivo*. (a) Survival of athymic mice bearing CAPAN-2 human pancreatic cancer cells treated with CORM-2 (35 mg/kg/day, i.p. administration). Data expressed as mean ± SD, graphs represent survival times (continuous line) and respective upper and lower confidence intervals (dashed lines), $n = 6$ for each group. (b) Immunohistochemical localisation and (c) quantification of CD31-positive vessels in CAPAN-2 tumours from mice after 14 days of treatment with CORM-2. (d) Tumour size in athymic mice bearing PaTu-8902 human pancreatic cancer cells exposed to CO (500 ppm CO for 1 h/day). $^*p < 0.05$ (CO treatment *vs.* control, based on RM ANOVA with Holm–Sidak *post hoc* testing). (e) Survival curve of athymic mice bearing PaTu-8902 human pancreatic cancer cells exposed to CO (500 ppm CO for 1 h/day). Data expressed as mean ± SD, graphs represent survival times (continuous line) and respective upper and lower confidence intervals (dashed lines), $n = 6$ for each group. ANOVA, analysis of variance; CO, carbon monoxide; CORM, carbon monoxide-releasing molecule; iCORM, inactive CORM; SD, standard deviation; ppm, parts per million.

and was even able to induce apoptosis in the CAPAN-2 pancreatic cancer cell line ($p < 0.05$, Fig. 1e).

Analysis of CO in the PaTu-8902 pancreatic cancer cells, exposed to 50 μmol/L of CORM-2, revealed a substantial increase in CO concentration within the cells (Fig. 3a) as well as the culture media (Fig. 3b).

3.2. CO inhibits pancreatic tumour growth in vivo

To confirm our *in vitro* data, the anticancer effects of CO were validated in an experimental animal cancer model, represented by athymic mice xenografted subcutaneously with human pancreatic cancer cells (CAPAN-2/PaTu-8902).

In the first model, athymic mice carrying CAPAN-2 xenografts were administered a dose of 35 mg/kg CORM-2 intraperitoneally, on a daily basis. Treatment with CORM-2 induced a significant increase in the survival rate, compared with the iCORM-2 treated control animals (37.7 ± 10.1 *vs.* 17.2 ± 2.1 days, $p = 0.0005$, Fig. 4a). Analysis of the capillary density of the tumour revealed a decrease in the number of CD31-positive vessels in CORM-2-treated, CAPAN-2 xenografted animals, compared with mice receiving iCORM-2 ($p < 0.01$, Fig. 4b and c); indicating that CORM-2 was able to substantially affect *de novo* angiogenesis. This finding was consistent with

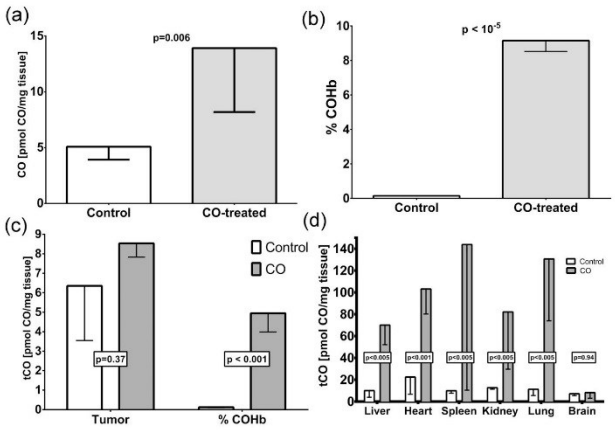

Fig. 5. The effect of CO exposure on its distribution in the body. (a) CO levels in tumour tissues, and (b) CO-haemoglobin levels in animals sacrificed immediately after the last exposure (day 14) to CO (500 ppm CO for 1 h/day). (c) CO levels in tumour tissue and CO-haemoglobin proportion, and (d) CO levels in various other organs of animals sacrificed 8 h after the last exposure (day 14) to CO (500 ppm CO for 1 h/day) control = tissues from animals unexposed to CO; $n = 6$ for each group. CO, carbon monoxide; SD, standard deviation; ppm, parts per million.

our *in vitro* data on CORM-2-induced suppression of Akt phosphorylation, since downstream signals from the Akt signalling pathway are known to contribute significantly to cancer neovascularisation [19].

Similarly, as in the *in vitro* studies, in order to verify whether CO released from the CORM-2 molecule was responsible for the observed antiproliferative/antiangiogenic effects of CORM-2, we also investigated the effect of CO *per se*. We assessed the tumour volume and survival in mice carrying subcutaneous human pancreatic ductal adenocarcinoma PaTu-8902 xenografts, which were treated with CO (500 ppm, 1-h exposure per day). CO-treated PaTu-8902 tumour-bearing mice showed a significant decrease in mean tumour volume ($p < 0.05$, Fig. 4d) and a two-fold increase in survival rate ($p = 0.004$, Fig. 4e), compared with the control mice exposed to air.

Analysis of CO levels in the tumour tissue, performed immediately after the last exposure to CO, demonstrated more than an 170% increase in CO concentrations at target tumours, compared with those of control mice ($p = 0.006$, Fig. 5a); this was paralleled by a marked increase in serum CO-haemoglobin levels ($p < 10^{-5}$, Fig. 5b). To assess the long-term distribution effects of CO exposure, we also assessed tissue levels of CO 8 h after the last exposure to the treatment gas. Consistent with its systemic effects on pancreatic cancer proliferation, CO concentrations in various organs, including subcutaneously xenografted pancreatic cancer, remained substantially high (Fig. 5c) even after this prolonged period of time, indicating the long-term systemic distribution of CO.

4. Discussion

For a long time, CO has been considered a toxic gas with potentially lethal consequences; however, it now appears to have multiple beneficial effects for human health. The importance of CO is underlined by the fact that substantial amounts of this gas is produced endogenously, as a result of the action of HMOX, which is among the most important stress enzymes in the human body [20].

Our *in vitro* and *in vivo* data show that exogenously administered CO, either in the form of CORM-2 or as direct CO exposure, acts as a potent inhibitor of pancreatic carcinogenesis. Indeed, in mice carrying human pancreatic xenografts, CO reduces tumour volume, limits tumour neovascularisation and profoundly prolongs survival. The doses of CO used in our *in vivo* study (500 ppm of CO for 1 h a day) are far below lethal doses for mice, which is equal to inhalation of 2400 ppm for 4 h [21]. This is also evidenced by the acceptable CO-haemoglobin levels of the CO-treated animals, which are even below the CO-haemoglobin concentrations achieved from clinically relevant CO dosages, using a FDA-approved CO inhalation device for human use [7]. Based on the very promising therapeutic potential of CO, the effects of CO inhalation at dosing regimens equivalent to those used in our work are currently being investigated in other clinical trials with patients suffering from pulmonary or intestinal diseases with safety and tolerability issues being investigated as well.[3]

Collectively, our data point to the antiproliferative nature of CO, which may genuinely have a chemotherapeutic/chemoadjuvant potential against certain cancers. CO along with NO and H_2S, belong to the potent bioactive gases having multiple, often shared, biological functions [22]. However, the observed antiproliferative action of CO appears to have the opposite effects to that of NO. NO generated following activation of endothelial nitric oxide synthase (NOS) promotes malignant progression [23] and VEGF-driven neovascularisation [24,25] through activation of the pro-survival Akt pathway [26], which is critical for ischemic and VEGF-mediated angiogenesis [19]. Positive inducible NOS expression correlates with increased microvessel density in human gastric cancer [27], although a dual role of NO in human carcinogenesis has been documented [28]. Based on our data, CO seems to inhibit Akt phosphorylation, which in turn is reflected by decreased neovascularization, as observed in our CORM-2 treated mice. This is in accord with the report by Becker et al. [29], which demonstrated a better prognosis for colon cancer patients overexpressing HMOX1 (responsible for endogenous CO production) in tumour tissues. Furthermore, Ferrando et al. demonstrated that HMOX1 overexpression in prostate cancer cells potently suppressed angiogenesis [30].

[3] www.clinicaltrials.gov [accessed 15.10.13].

Nevertheless, CO seems to contribute to the suppression of carcinogenesis by several additional mechanisms, as shown in the CO-induced inhibition of photocarcinogenesis [31] or migration of hepatoblastoma HepG2 cells [32]. CO derived from CORM-3 was also shown to interfere with the p38 MAPK signalling pathway in endothelial cells [33], which is consistent with our data on the CO-induced inhibition of Akt phosphorylation in pancreatic cancer cells. Furthermore, CO, a potent activator of guanylate cyclase [34], may also modulate cellular proliferation *via* increased cGMP production [35]. Additionally, for the first time we can report pharmacokinetic data of CO inhaled by experimental animals demonstrating a clinically important CO distribution within the various organs and tissues of the mouse body, including subcutaneously xenografted tumours, even after a prolonged period of time. Consistent with these data, we have also validated the efficient distribution of CO within pancreatic cancer cells exposed to the CORM-2 molecule. These kinetic data support the idea that CO might possibly be used in clinical settings.

In conclusion, we found that CO in relatively low (but clinically relevant and applicable) doses acts as a potent anticancer agent. These data suggest that CO could potentially be exploited as a novel chemotherapeutic/chemoadjuvant gas for inhibiting tumour growth.

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Conflict of interest statement

None to report.

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Annex 4

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Research Article

Isolated Silymarin Flavonoids Increase Systemic and Hepatic Bilirubin Concentrations and Lower Lipoperoxidation in Mice

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Bilirubin is considered to be one of the most potent endogenous antioxidants in humans. Its serum concentrations are predominantly affected by the activity of hepatic bilirubin UDP-glucuronosyl transferase (UGT1A1). Our objective was to analyze the potential bilirubin-modulating effects of natural polyphenols from milk thistle (*Silybum marianum*), a hepatoprotective herb. Human hepatoblastoma HepG2 cells were exposed to major polyphenolic compounds isolated from milk thistle. Based on *in vitro* studies, 2,3-dehydrosilybins A and B were selected as the most efficient compounds and applied either intraperitoneally or orally for seven days to C57BL/6 mice. After, *UGT1A1* mRNA expression, serum, intrahepatic bilirubin concentrations, and lipoperoxidation in the liver tissue were analyzed. All natural polyphenols used increased intracellular concentration of bilirubin in HepG2 cells to a similar extent as atazanavir, a known bilirubinemia-enhancing agent. Intraperitoneal application of 2,3-dehydrosilybins A and B (the most efficient flavonoids from *in vitro* studies) to mice (50 mg/kg) led to a significant downregulation of *UGT1A1* mRNA expression ($46 \pm 3\%$ of controls, $p < 0.005$) in the liver and also to a significant increase of the intracellular bilirubin concentration (0.98 ± 0.03 *vs.* 1.21 ± 0.02 nmol/mg, $p < 0.05$). Simultaneously, a significant decrease of lipoperoxidation ($61 \pm 2\%$ of controls, $p < 0.005$) was detected in the liver tissue of treated animals, and similar results were also observed after oral treatment. Importantly, both application routes also led to a significant elevation of serum bilirubin concentrations ($125 \pm 3\%$ and $160 \pm 22\%$ of the controls after intraperitoneal and oral administration, respectively, $p < 0.005$ in both cases). In conclusion, polyphenolic compounds contained in silymarin, in particular 2,3-dehydrosilybins A and B, affect hepatic and serum bilirubin concentrations, as well as lipoperoxidation in the liver. This phenomenon might contribute to the hepatoprotective effects of silymarin.

1. Introduction

Bilirubin, the end product of heme catabolism in the systemic circulation, is a potent antioxidant substance [1]. Despite the fact that for decades bilirubin has been considered a toxic catabolic waste product and an ominous sign of liver dysfunction, its role as a powerful protective molecule has increasingly been recognized [2]. *In vitro* and *in vivo* studies have shown that bilirubin may suppress the oxidation of lipids [1] and has anti-inflammatory [3], antiproliferative [4], antigenotoxic [5], antimutagenic [6], or even anti-aging properties [7]. Interestingly, bilirubin has been reported as a potent peroxisome proliferator-activated receptor-α (PPARα) agonist, thus acting as a real endocrine molecule, with all potential clinical consequences [8]. The clinical evidence is even more important. Bilirubin, when only mildly elevated, has been demonstrated to protect from a wide array of oxidative stress-related diseases, including cardiovascular diseases, certain cancers, and autoimmune or neurodegenerative diseases [2, 9]. In fact, substantial protective effects of

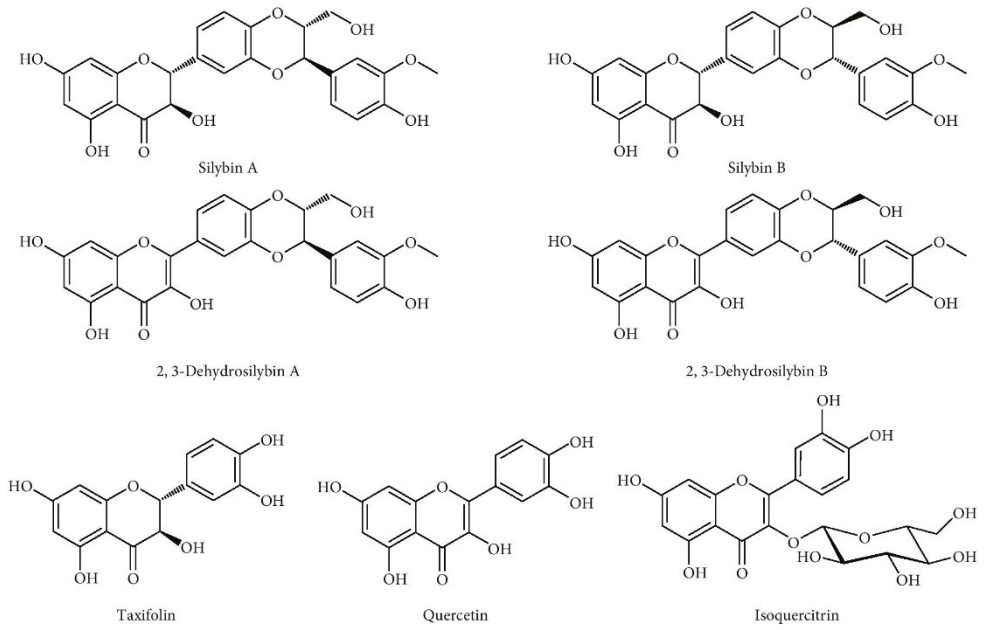

FIGURE 1: Structures of flavonolignans of the silymarin complex and related flavonols.

mild unconjugated hyperbilirubinemia, as seen in subjects with Gilbert syndrome (benign hyperbilirubinemia), have been reported for atherosclerotic diseases in particular [10].

Bilirubin production is dependent on heme oxygenase (HMOX) activity, but systemic bilirubin concentrations are predominantly affected by hepatic bilirubin UDP-glucuronosyl transferase (UGT1A1), its biotransforming enzyme [11]. Partial inhibition of bilirubin glucuronosylation was proposed as a wise strategy used to induce "iatrogenic" Gilbert syndrome [12]. This indeed was demonstrated as a "side effect" of several drugs used in various indications, typically for atazanavir, whose administration is often associated with mildly elevated concentrations of unconjugated bilirubin. Surprisingly, hyperbilirubinemia induced by atazanavir was reported to decrease markers of oxidative stress [13] and cardiometabolic risk factors [14], as well as endothelial functions [15], but safer approaches are certainly needed.

Since xenobiotics used in clinical medicine are often associated with potentially severe side effects, natural compounds interfering with the UGT1A1 hepatic biotransformation system seem a better strategy to induce mild hyperbilirubinemia. Based on the scarce data reported in the literature (mainly as the result of investigating potential nutraceuticals/herb-drug interactions), it seems that flavonoids from silymarin might modulate this enzyme. This assumption is based on the fact that many herbal extracts including silymarin, a seed extract of milk thistle (*Silybum marianum* (L.) Gaertn.), are rich in phenolic phytochemicals that are substrates for UGT1A1 or even exhibit UGT1A1-inhibiting activities [16–18]. Indeed, therapy for prostate cancer patients with high doses of silybin (silibinin) has been associated with unconjugated hyperbilirubinemia, which was considered by the authors as an adverse effect of such treatment [19]. Similar findings were also reported in hepatitis C patients receiving silybin therapy [20–23]. Although most of the experimental reports as well as some clinical data suggest its beneficial role, silymarin is generally considered to have a negligible importance clinically [24]. There are many possible reasons: one of them being the poorly defined content of the active ingredients and also the improperly characterized biological properties of individual pure flavonoids in the silymarin complex [25, 26]. Silymarin is a mixture of 5 major flavonolignans (silybins A and B, isosilybin A, silychristin A, and silydianin) plus their precursor taxifolin, as well as other minor polyphenolic compounds (Figure 1) [27]. Among them, the 2,3-dehydroflavonolignans such as 2,3-dehydrosilybins A and B possess potent biological activities [28–32].

Thus, the aim of our study was to investigate the potential bilirubin-modulating effects of natural polyphenols present in milk thistle and related compounds.

2. Materials and Methods

2.1. Chemicals. Silymarin (containing 13.0% of silybin A, 17.9% silybin B, 14.7% silychristin A, 9.3% silychristin B +silydianin, 8.9% isosilybin A, 6.8% isosilybin B, 3.0%

taxifolin, 1.9% 2,3-dehydrosilybin, 0.5% 2,3-dehydrosilychristin, 6.5% of other nonidentified 2,3-dehydroflavonolignans, plus 17.5% of yet other substances (probably polymers, for details of the analysis, see the Supplementary Data and Supplementary Figures 1–3), silybin AB (approximately an equimolar mixture of silybin A and silybin B), quercetin, and rutin (quercetin-3-*O*-β-rutinoside)) was all purchased from Sigma-Aldrich (St. Louis, MO, USA); atazanavir was obtained from Santa Cruz (Santa Cruz Biotechnology, Dallas, TX, USA). Silybin A and silybin B were isolated from silymarin (purchased from Liaoning Senrong Pharmaceutical, Panjin, China; batch no. 120501), as described in [33]. 2,3-Dehydrosilybin, 2,3-dehydrosilybin A, and 2,3-dehydrosilybin B were prepared by oxidation of silybin, silybin A, and silybin B, respectively [34]. Taxifolin hydrate (92%) was purchased from Amagro (Prague, CZ), and isoquercitrin (quercetin-3-*O*-glucoside, 97%) was prepared from rutin (Sigma-Aldrich) using thermophilic α-L-rhamnosidase from *Aspergillus terreus* heterologously expressed in *Pichia pastoris* [35]. The deconjugation enzymes β-glucuronidase and sulfatase from *Helix pomatia* were obtained from Sigma-Aldrich.

2.2. Cell Cultures. The HepG2 human hepatoblastoma cell line was used for the *in vitro* studies (ATCC, Manassas, VA, USA). Cells were cultured in MEM Eagle medium, containing 10% of fetal bovine serum, in 75 cm^2 culture flasks, at 37°C, in a 5% CO_2 atmosphere. For an estimation of the intracellular bilirubin concentration, the cells were plated in 10 cm Petri dishes and treated with natural polyphenols dissolved in DMSO (vehicle; 0.66%, *v*/*v*) for 24 h. For qPCR measurement, the cells were cultured in 24-well plates and treated with natural flavonoids dissolved in DMSO (vehicle; 0.66%, *v*/*v*) for 4 h.

The cell lines were authenticated at ATCC by STR profiling before distribution and also reauthenticated at the end of the study by an external laboratory (Generi Biotech, Hradec Králové, Czech Republic).

2.3. MTT Cytotoxicity Assays. The MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) reduction assay in a 96-well format was used for the determination of the cytotoxicity of the compounds tested. HepG2 cells were seeded into a 96-well plate at a density of 1×10^5 cells/well. Following 24 h incubation, the cells were treated for 24 h with natural flavonoids at concentrations of 0.1-200 μM dissolved in DMSO (vehicle; 0.66%, *v*/*v*). MTT assay was measured spectrophotometrically at 540 nm (TECAN, Schoeller Instruments s.r.o., Prague, Czech Republic).

2.4. RNA Isolation and Real-Time PCR. Total mRNA was isolated using a 5 Prime PerfectPure RNA Cultured Cell Kit and a 5 Prime PerfectPure RNA Tissue Kit (Eppendorf, Germany); and cDNA was generated using a High Capacity RNA-to-cDNA Master Mix (Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instructions. qPCR for the cell culture was performed using the SYBR™ Select Master Mix (Life Technologies). For each reaction, the final 20 μL volume was comprised of 10 μL of SYBR Green PCR Mix, 2 μL of each primer (Supplementary Table 1), and 6 μL of a 1/5 dilution of the RT products. qPCR for the liver tissue was performed using a TaqMan® Gene Expression Assay Kit (Life Technologies). Threshold cycle (Ct) values were analyzed using the comparative Ct ($\Delta\Delta$Ct) method as recommended by the manufacturer (Applied Biosystems, Foster City, CA, USA). The data were normalized to the expression of hypoxantin phosphoribosyl transferase (*HPRT*) and expressed as the multiplicity change from the control levels.

2.5. Serum Markers of Liver Damage. Hepatic enzyme (alanine aminotransferase (ALT), aspartate aminotransferase (AST), and alkaline phosphatase (ALP)) activities were determined on an automatic analyzer (Modular Analyzer, Roche Diagnostics GmbH, Germany) using standard assays.

2.6. Determination of Bilirubin. For the determination of bilirubin in HepG2 cells and liver tissue, the biological materials were sonicated on ice and then extracted with methanol/chloroform/hexane 10/5/1 (*v*/*v*/*v*) against PBS buffer (pH 6.2). The lower organic phase was subsequently extracted into 50 μL of carbonate buffer (pH 10) in hexane. The resulting polar droplet was loaded onto a C-8 reverse phase column (Luna 3 μm, 150 × 4.6 mm, Phenomenex, Torrance, CA, USA), and bilirubin was determined using an HPLC Agilent 1200 with a diode array detector (Agilent, Santa Clara, CA, USA) as described earlier [36, 37]. The concentration of bilirubin was calculated in nmol/g of wet tissue or pmol/mg of protein for tissue samples or cell cultures, respectively.

For determination of bilirubin in serum, the LC-MS/MS method was used. Ten μL of serum was mixed with 2.5 μL of internal standard (mesobilirubin, $c = 20$ μmol/L) and deproteinized by 50 μL of 1% BHT in methanol, 50 μL of 0.4% ascorbic acid in methanol, and 200 μL of methanol (pH 11). Five μL of the resulting supernatant was loaded on a Poroshell 120 EC-C18 2.1 μm 3.0 × 50 mm column (Agilent), with a gradient of 1 mM NH_4F in water (A) and methanol (B) as follows: 60% of B was changed in 5 minutes to 100% of B, with a flow rate of 0.4 mL/min and was kept at 100% of B for 3 minutes, with the gradient of flow rate at 0.5 mL/min. The flow was then maintained at 0.5 mL/min until the end of the gradient program. Phase B was changed back to 60% in 0.1 minutes and was kept at 60% until the end of the program at 10 minutes. The mass spectra were recorded on an Agilent 6470 LC/QQQ (Agilent) LC-MS/MS device with electrospray ionization and multiple reaction monitoring in a positive mode. Conditions of the electrospray ionization source, gas flows, and potentials of the mass spectrometer were manually tuned for high sensitivity and a low signal-to-noise ratio of the analytes: ion spray voltage, 3000 V; source temperature, 250°C; heater gas, 8 L/min; nebulizer, 300 psi; sheath gas temperature, 400°C; sheath gas flow, 12 L/min; and nozzle voltage, 500 V. Within the total scan time of 10 min, m/z 585.3 → 299.1, 16 eV collision energy (CE) at 5.96 min was monitored for bilirubin and m/z 589.3 → 301.2, 20 eV CE at 6.084 min for mesobilirubin.

2.7. HMOX Activity Assay. Twenty μL of liver sonicate (10%) was incubated for 15 min at 37°C in CO-free septum-sealed vials containing 20 μL of 150 μM heme and 20 μL of 4.5 mM NADPH, as previously described [38]. Blank reaction vials contained potassium phosphate buffer in place of NADPH. 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, Mountain View, CA, USA). HMOX activity was calculated as pmol CO/h/mg fresh weight and expressed as percentage of the control.

2.8. UGT1A1 Inhibition Assay. The UGT activity in the microsomal samples was determined by a UGT-Glo™ assay kit according to the manufacturer's instructions (Promega, Madison, WI, USA). Briefly, microsomes with recombinant UGT1A1 (0.0125 mg/mL) were incubated with 16 mM UDPGA and 20 μM multienzyme substrate with increasing concentrations of 2,3-dehydrosilybins A and B at 37°C for 30 min. Then, 40 μL of reconstituted luciferin detection reagent containing D-cysteine was added, and the luminescent signal was allowed to stabilize for 20 min at room temperature. Luminescence was read on a Synergy II plate reader (BioTek, VT, USA). The data were analyzed using the curve fitting method with GraphPad Prism (GraphPad Software, San Diego, CA).

2.9. Malondialdehyde Determination. Malondialdehyde (MDA) in the tissue homogenates was measured according to the method described by Wills [39], with some modifications. A 200 μL aliquot of 10% tissue homogenate was mixed with 2 mL of the thiobarbituric acid-trichloroacetic acid reagent (0.375 and 15%, respectively). The mixture was heated on a water bath at 95°C for 20 min. The solution was then cooled to room temperature. The reaction product (thiobarbituric acid-MDA complex) was extracted by adding 3 mL of *n*-butanol to the above solution. The absorbance of the pink-colored extract in *n*-butanol was measured at 532 nm using a spectrophotometer (Sunrise, Tecan, USA). The amount of MDA was calculated using the molar extinction coefficient of $1.56 \times 10^5\ M^{-1} \times cm^{-1}$ and expressed as a percentage of the control.

2.10. 2,3-Dehydrosilybin Determination in Sera. 2,3-Dehydrosilybin concentrations in serum samples were determined after deconjugation of phase II conjugates (sulfates and glucuronide). The activity of the deconjugation enzymes (β-glucuronidase and sulfatase) was checked on the selected glucuronides and sulfates prior to analysis. All the samples were measured in duplicate.

The conjugates in serum samples were deconjugated in acetate buffer (50 mM, pH 5.0) by adding β-glucuronidase (440 U) with sulfatase (35 U), incubated for 2 h at 37°C and 600 rpm. The reaction mixture was then freeze-dried, and an internal standard (2,3-dehydrosilychristin, 4.8 μg/mL, 150 μL, in acetonitrile/DMSO 1 : 1) was added. Samples were incubated (1 h, 37°C, 600 rpm) and centrifuged (15 min), and the supernatant was injected into a LC-MS (injection volume 25 μL).

2.10.1. LC-MS Conditions. LC-MS chromatograms and mass spectra were obtained using the Shimadzu Prominence system consisting of a DGU-20A3 mobile phase degasser, two LC-20AD solvent delivery units, a SIL-20AC cooling autosampler, a CTO-10AS column oven with the SPD-M20A diode array detector, plus a LCMS-2020 mass detector with a single quadrupole, equipped with an electrospray ion source (Shimadzu, Kyoto, Japan).

The LC-MS of the serum samples and standard solutions of 2,3-dehydrosilybin were measured on a Chromolith RP-18e (100 × 3 mm) column (Merck) and Chromolith RP-18e (5 × 4.6 mm) precolumn (Merck) (mobile phase: A = 5% acetonitrile, 0.1% HCOOH; B = 80% acetonitrile, 0.1% HCOOH; gradient: 0 min 20% B, 5 min 90% B, 6 min 90% B, and 8-10 min 20% B; flow rate: 0.4 mL/min, 25°C). The concentration of 2,3-dehydrosilybin in the serum was calculated using a calibration curve. The MS parameters were as follows: ESI interface voltage, 4.5 kV; detector voltage, 1.15 kV; nebulizing gas flow, 1.5 mL·min^{-1}; drying gas flow, 15 mL·min^{-1}; heat block temperature, 200°C; DL temperature, 250°C; negative scan mode, 478.8-481.0 *m/z*; and software, LabSolutions ver. 5.75 SP2 (Shimadzu, Kyoto, Japan).

2.11. Animal Studies. Female C57BL/6 mice (n = at least 6 in each group, 8 w old) obtained from Velaz (Prague, Czech Republic) had access to both water and a standard diet *ad libitum*. An equimolar mixture of dehydrosilybins A and B dissolved in DMSO (vehicle; 5%, *v/v*) was applied intraperitoneally, and individual dehydrosilybins were applied orally for seven days at a dose of 50 mg/kg b.wt. After 24 h of the last application, the mice were sacrificed and blood from their *superior vena cava* was collected for further analyses. The livers were cleaned and stored as pieces in nitrogen until used. 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 until the RNA isolation was performed.

All animal studies met the criteria for the care and use of animals in experiments and were approved by the Animal Research Committee of the 1st Faculty of Medicine, Charles University, Prague.

2.12. Statistical Analyses. All data are expressed as mean ± SEM. Depending on their normality, the data were analyzed either by the Student *t*-test or the Mann–Whitney rank sum test and Kruskal-Wallis ANOVA with Dunn's correction. Differences were considered statistically significant when P values were less than 0.05.

3. Results

3.1. The Effect of Silymarin Flavonoids and Related Compounds on HMOX and UGT1A1 Expressions and Activities in HepG2 Cells. In our *in vitro* screening studies, focused on evaluation of the possible modulating effect of silymarin flavonoids and related compounds on *HMOX1* and *UGT1A1* expressions, a wide array of compounds were used (Figure 1). Based on the MTT assays (Table 1), *HMOX1* and *UGT1A* mRNA expression analyses were performed

Table 1: Inhibitory concentrations of individual silymarin flavonoids and related compounds.

Compound	IC_{50} (μM)
Silybin A	>200
Silybin B	>200
Silybin AB	>200
Isosilybin A	148 ± 3
Isosilybin B	185 ± 4
2,3-Dehydrosilybin A	>200
2,3-Dehydrosilybin B	>200
Silychristin	>200
2,3-Dehydrosilychristin	>200
Silydianin	>200
2,3-Dehydrosilydianin	>200
Isoquercitrin	115 ± 4
Quercetin	105 ± 3
Taxifolin	>200

HepG2 cells were treated for 24 h with natural flavonoids in concentrations of 0.1-200 μM. IC: inhibitory concentration, measured by the MTT test.

with the nontoxic plus 1/2 of nontoxic concentrations for HepG2 cells.

Significant *HMOX1* mRNA overexpression was observed for quercetin and isoquercitrin; on the other hand, *HMOX1* mRNA downregulation was present after exposure to 2,3-dehydrosilybins A and B (Figure 2). More importantly, the activity of HMOX was upregulated not only by quercetin and isoquercitrin but also after exposure to the silymarin complex *per se*, as well as silybins A and B. Downregulation of *HMOX1* mRNA expression by 2,3-dehydrosilybins A and B (Figure 2) was also reflected by decreased HMOX activity (Figure 3(a)).

Significant underexpression of *UGT1A1* mRNA was noted after exposure of HepG2 cells to the equimolar mixture of silybins A and B, to quercetin, isoquercitrin, taxifolin, and 2,3-dehydrosilybins A and B (Figure 2). These results were reflected by the inhibition of UGT1A1 activity, as determined for 2,3-dehydrosilybins A and B (IC_{50} values of 2.1 ± 0.2 and 4.1 ± 0.2 μmol/L, respectively; Figure 3(b)).

3.2. The Effect of Silymarin Flavonoids and Related Compounds on Intracellular Concentrations of Bilirubin in HepG2 Cells. To investigate whether the effects of silymarin flavonoids and related compounds on HMOX and UGT1A1 are translated into phenotypic changes, we analyzed bilirubin concentrations within the HepG2 cells after 24 h exposure to individual compounds.

Most of silymarin flavonoids and related compounds significantly elevated intracellular bilirubin (Figure 4) in a dose-dependent manner (data not shown). Interestingly, the increase of intracellular bilirubin concentrations caused by exposure of both 2,3-dehydrosilybins A and B was even higher than that caused by atazanavir, a known inhibitor of UGT1A1 [40] (Figure 4).

Based on these *in vitro* results, 2,3-dehydrosilybins A and B were selected for the *in vivo* studies.

3.3. The Effect of 2,3-Dehydrosilybins A and B on Bilirubin Metabolism in Mice

3.3.1. Concentrations of 2,3-Dehydrosilybins in Sera. Since 2,3-dehydrosilybins have a low solubility in water and the poor oral bioavailability might contribute to the low clinical efficiency of silymarin in humans, we first measured concentrations of 2,3-dehydrosilybins in sera to verify whether they pass into the systemic circulation. Use of both application routes (*i.e.*, intraperitoneal as well as oral administration of 2,3-dehydrosilybins (50 mg/kg b.wt.)), after 24 h, resulted in a substantial appearance in the systemic blood, reaching serum concentrations up to 300 ng/mL (0.62 μmol/L) (Figure 5).

3.3.2. Hepatic HMOX1 and UGT1A1 mRNA Expressions. Then we analyzed the effect of 2,3-dehydrosilybin administration on *HMOX1* and *UGT1A1* mRNA expressions in the livers of treated mice. Treatment with an equimolar mixture of 2,3-dehydrosilybins A and B (50 mg/kg b.wt.) administered intraperitoneally for 7 days led to a significant downregulation of *UGT1A1* mRNA expression in the liver (57 ± 19% of the controls) with no change in *HMOX1* expression (Figure 6(a)). Similar results were observed for oral treatment with 2,3-dehydrosilybin A (downregulation of *UGT1A1* mRNA expression to 55 ± 26% of the control, Figure 6(b)); while no effect was demonstrated for 2,3-dehydrosilybin B. None of the individual dehydrosilybins had any effect on *HMOX1* expression (Figure 6(b)).

3.3.3. Intracellular and Systemic Bilirubin Concentrations. Intraperitoneal application of 2,3-dehydrosilybins A and B mixture significantly increased intracellular concentrations of bilirubin in the liver tissue (to 149 ± 24%, $p < 0.05$, Figure 6(c)). An even higher increase was observed after oral treatment of 2,3-dehydrosilybin A (to 236 ± 28%, $p < 0.05$; Figure 6(d)), whereas treatment with 2,3-dehydrosilybin B only led to a moderate but not significant elevation (Figure 6(d)).

Neither intraperitoneal nor oral administration of 2,3-dehydrosilybins caused any increase in markers of liver damage (ALT, AST, and ALP activities; data not shown).

Consistent with the results of intrahepatic bilirubin concentrations, intraperitoneal administration of the 2,3-dehydrosilybins A and B mixture also significantly elevated serum bilirubin concentrations (by 27%; 0.96 ± 0.09 vs. 1.22 ± 0.35 μM; Figure 6(e)). Even a more pronounced elevation of serum bilirubin concentrations was observed after oral treatment with 2,3-dehydrosilybin A (by 44%; 0.96 ± 0.18 vs. 1.38 ± 0.29 μM; Figure 6(f)). Treatment with 2,3-dehydrosilybin B did not cause any changes in serum bilirubin concentrations (Figure 6(f)).

3.3.4. Hepatic Lipoperoxidation. Treatment of mice with 2,3-dehydrosilybins had consistent inhibitory effects on lipid peroxidation in the liver tissue. MDA concentrations in the liver tissue were significantly reduced after intraperitoneal

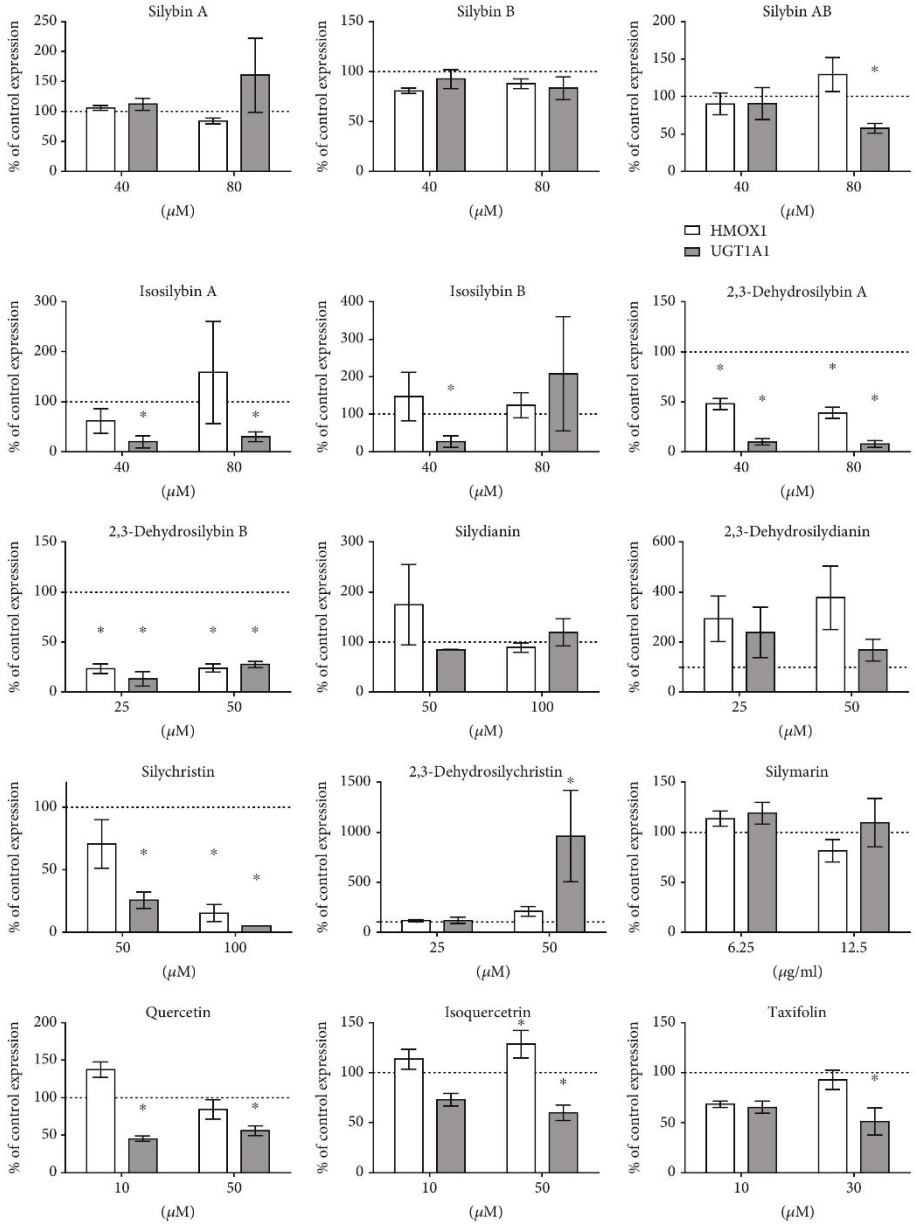

FIGURE 2: The effect of silymarin flavonoids and related compounds on *HMOX1* and *UGT1A1* expressions in HepG2 cells. mRNA expressions were analyzed after 4 h exposure of HepG2 cells to individual flavonolignans (in corresponding nontoxic concentrations). Data are expressed as percentage of control values. $^*P < 0.05$.

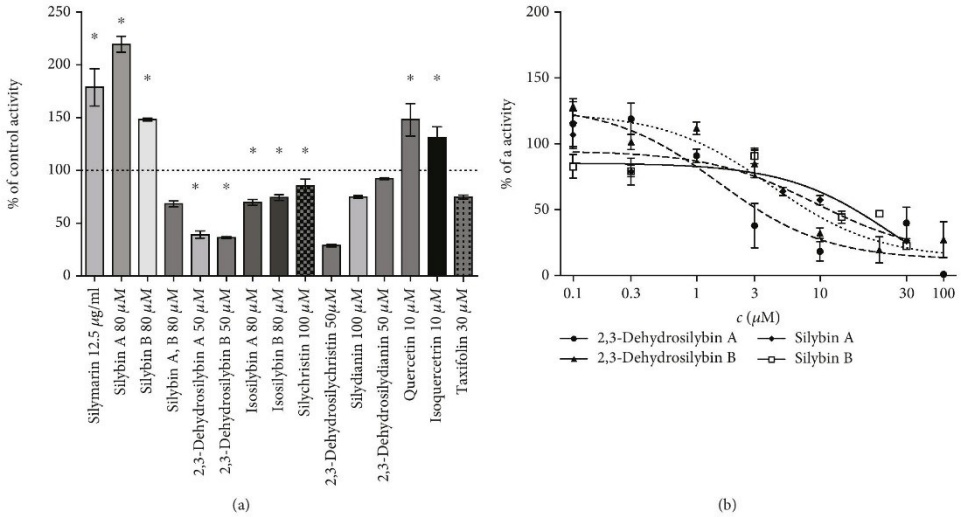

Figure 3: The effect of silymarin flavonoids and related compounds on (a) HMOX and (b) UGT1A1 activities in HepG2 cells. HMOX and UGT1A1 activities were analyzed after 24 h exposure of HepG2 cells to individual flavonolignans. HMOX activity was calculated as pmol CO/h/mg fresh weight and expressed as a percentage of the control values. UGT1A1 enzyme activity was based on relative light unit values and expressed as a percentage of the remaining activity. $^{*}P < 0.05$.

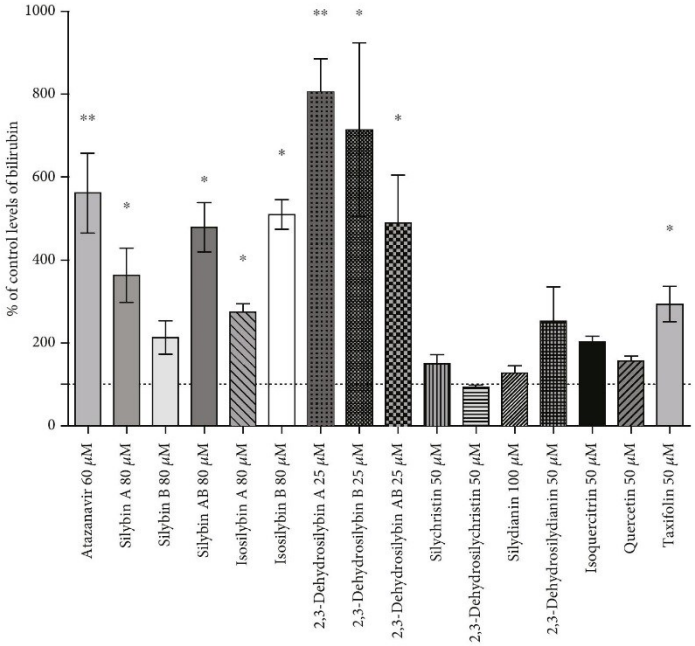

Figure 4: The effect of silymarin flavonoids and related compounds on intracellular concentrations of bilirubin in HepG2 cells. Control values are expressed as 100% and correspond to 2 pmol of bilirubin per mg of protein. $^{*}P < 0.05$; $^{**}P < 0.005$.

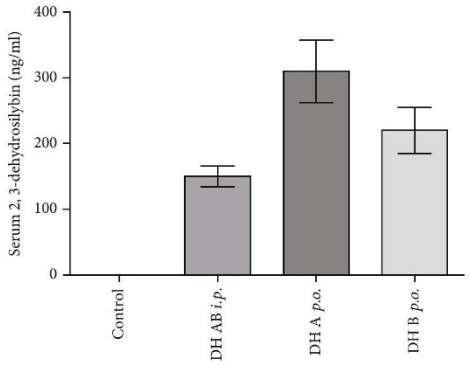

FIGURE 5: Serum concentrations of 2,3-dehydrosilybins after intraperitoneal and oral administration. 2,3-Dehydrosilybins (DH) were administered at a dose of 50 mg/kg b.wt. *i.p.*: intraperitoneal; *p.o.*: oral; b.wt.: body weight.

application of the mixture of 2,3-dehydrosilybins (to 70 ± 7% of the control; Figure 7(a)), and the same effect was also observed after oral administration of both 2,3-dehydrosilybins A and B (61 ± 15% and 64 ± 13% of the control, respectively; Figure 7(b)).

4. Discussion

Bilirubin, a bile pigment, for decades considered only an ominous sign of liver diseases, has been revisited as a potent antioxidant, immunosuppressive, and cytoprotective agent [2, 9, 10, 41]. Even single micromolar elevations of systemic concentrations of bilirubin, still within the physiological range, are associated with a substantial decreases of the risks of cardiovascular, cancer, and inflammatory diseases [42–44], and this association is clearly evident in subjects with Gilbert syndrome, characterized with mild systemic elevations of unconjugated bilirubin [9, 45]. This led to the suggestion to induce Gilbert syndrome iatrogenically in order to suppress the development of oxidative stress-related diseases [12].

As reported in our recent study, due to a dynamic equilibrium, a correlation between systemic and intracellular concentrations of bilirubin exists in the liver (and also probably in other organs and tissues) [37]. Thus, it is important to monitor intrahepatic concentrations of bilirubin when assessing potential hepatoprotective effects, in particular when considering the fact that hepatic UGT1A1 is the major enzyme affecting systemic concentrations of bilirubin [11]. Hepatic UGT1A1 is a biotransforming enzyme important not only for bilirubin but also for a variety of other endogenous substances as well as xenobiotics, including natural agents often used as nutraceuticals, including the flavonolignans and flavonols of the silymarin complex. In fact, inhibitory effects towards UGT1A1 were reported for silybin, a major flavonoid constituent of milk thistle extract [46]; indeed, the therapy with this substance has also been associated with marked unconjugated hyperbilirubinemia [19]. A significant increase in serum bilirubin levels was also reported in other human studies on patients with chronic hepatitis C receiving silybin therapy [20–23]. These clinical data are consistent with our findings, *i.e.*, increased intracellular as well as systemic concentrations of bilirubin upon exposure to silymarin flavonoids. It is important to emphasize that these effects differed substantially among individual flavonoids—dehydrosilybins A and B being the most efficient.

Despite generally low bioavailability of silymarin flavonoids, effective serum concentrations of both 2,3-dehydrosilybins A and B were detected in our study (up to 300 ng/mL (Figure 5), corresponding to concentrations of 0.62 μmol/L). This value entirely fits to the mean peak plasma concentration of silybin, reached after oral administration of a 700 mg dose of silymarin (containing approximately 250 mg of silybin), which was 0.6 μmol/L [46]. Very similar plasma silybin concentrations were reported in another human study (0.4, 1.4, and 4 μmol/L after 360, 720, or 1440 mg of silybin administered daily for 7 days), with corresponding concentrations in the liver tissue [47], as well as in the studies by Barzaghi et al. (silybin plasma levels of 0.38 μmol/L after administration of 240 mg of silybin given daily for 7 consecutive days) [48] and Wen et al. (plasma concentrations of 0.8 μmol/L after oral administration of 600 mg of silymarin) [49].

In addition, silybin was demonstrated to potently inhibit UGT1A1, with the IC_{50} value correlating with the clinically relevant plasma concentrations [46]. Similar inhibitory concentrations were also reported by others for other silymarin flavonoids, although 2,3-dehydrosilybins were not tested in these studies [50]. These data are consistent with our findings, with the UGT1A1 IC_{50} value for 2,3-dehydrosilybin A = 2.1 μmol/L and the mean serum concentration of 0.62 μmol/L. Importantly, we found the UGT1A1 IC_{50} value for 2,3-dehydrosilybin A even lower than that reported for atazanavir (2.3 μmol/L), the well-known hyperbilirubinemia-inducing drug [40] indicating the high bilirubinemia-enhancing potential of this flavonolignan.

Although the UGT1A1 IC_{50} values of 2,3-dehydrosilybin A are slightly higher than those reported for silybin, 2,3-dehydrosilybin A most efficiently increased both the hepatic intracellular and systemic concentrations of bilirubin. The reason for this might be that 2,3-dehydrosilybin A also affects other mechanisms implicated in hepatic bilirubin metabolism. Such an example may include the modulation of the basolateral bilirubin organic anion-transporting proteins OATP1B1/3, which are also inhibited by silymarin flavonoids [51].

2,3-Dehydrosilybins, together with other 2,3-dehydroflavonolignans, belong to the minor flavonoids of the silymarin complex, usually accounting for 1-2% of all flavonolignans (1.8% in the preparation used in the present work; Supplementary Figure 3). Their biological effects, however, might be of real clinical importance [28]. This is supported by a recent observation that 2,3-dehydrosilybins A/B significantly suppressed oxidative stress in *C. elegans*

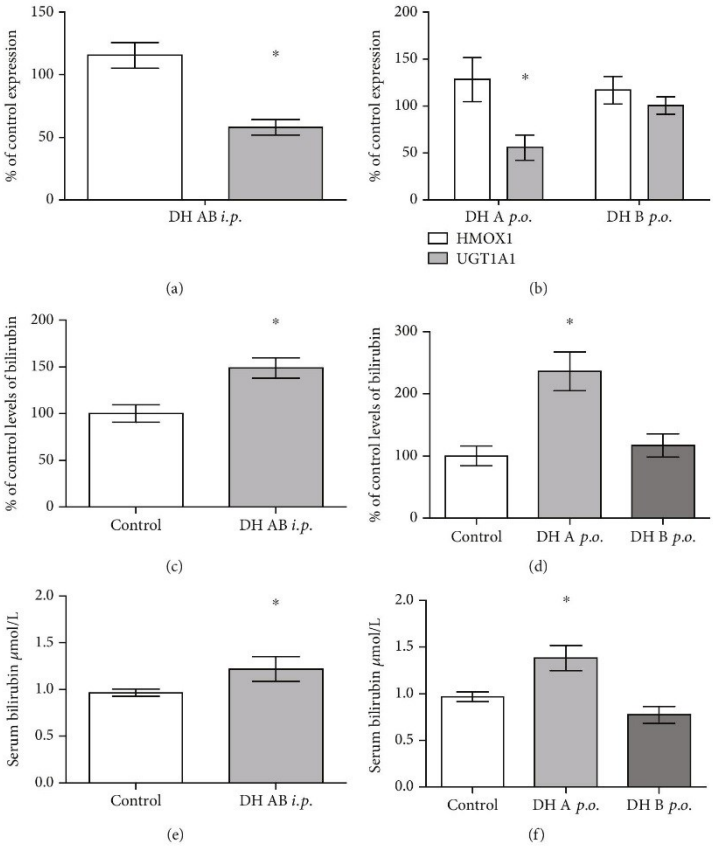

FIGURE 6: The effect of 2,3-dehydrosilybins on hepatic *HMOX1* and *UGT1A1* mRNA expressions and intrahepatic and systemic bilirubin concentrations. (a) *HMOX1* and *UGT1A1* mRNA expressions in mouse livers after intraperitoneal administration of a mixture of 2,3-dehydrosilybins A and B (50 mg/kg b.wt. for 7 days). (b) *HMOX1* and *UGT1A1* mRNA expressions in mouse livers after oral administration of either 2,3-dehydrosilybin A or B (50 mg/kg b.wt. for 7 days). (c) Intrahepatic bilirubin concentrations after intraperitoneal administration of a mixture of 2,3-dehydrosilybins A and B (50 mg/kg b.wt. for 7 days). (d) Intrahepatic bilirubin concentrations after oral administration of either 2,3-dehydrosilybin A or B (50 mg/kg b.wt. for 7 days). (e) Systemic bilirubin concentrations after intraperitoneal administration of a mixture of 2,3-dehydrosilybins A and B (50 mg/kg b.wt. for 7 days). (f) Systemic bilirubin concentrations after oral administration of either 2,3-dehydrosilybin A or B (50 mg/kg b.wt. for 7 days). $^*P < 0.05$. b.wt.: body weight.

resulting in lifespan extension [32]. Although the effect on bilirubin metabolism was not investigated in this study, it is important to note that the antiaging effects of bilirubin have been demonstrated in another of our studies [7]. In this respect, it is also important to emphasize the antioxidant effects of 2,3-dehydrosilybins observed in our study. Indeed, decreased MDA concentrations in the livers of mice treated with 2,3-dehydrosilybins might have been mediated, at least partially, *via* increased intracellular bilirubin concentrations. It is thus likely that these antioxidant effects are not directly related to antioxidant activities of the flavonolignans per se, but rather parahormetic action is more important [52].

5. Conclusions

Natural silymarin flavonolignans contained in milk thistle and related flavonols, in particular 2,3-dehydrosilybins A and B, affect hepatic and serum bilirubin concentrations, as well as lipoperoxidation in the liver. This phenomenon might contribute to the hepatoprotective effects of silymarin observed in many, although not all clinical studies.

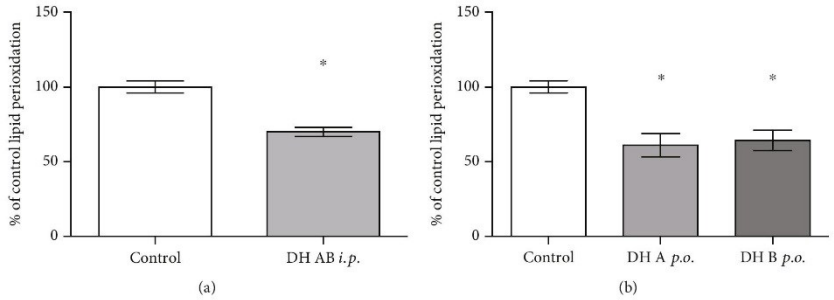

FIGURE 7: The effect of intraperitoneal and oral administration of 2,3-dehydrosilybins on lipoperoxidation in the liver tissue. (a) MDA concentrations in the mouse liver tissue after intraperitoneal administration of a mixture of 2,3-dehydrosilybins A and B (50 mg/kg b.wt. for 7 days). (b) MDA concentrations in the mouse liver tissue after oral administration of either 2,3-dehydrosilybin A or B (50 mg/kg b.wt. for 7 days). Data are expressed as a percentage of the control values. $^*P < 0.05$. b.wt.: body weight.

Modulation of bilirubin metabolism by well-defined natural polyphenols can represent a safe chemopreventive approach against oxidative stress-mediated diseases including atherosclerosis, cancer, diabetes, or inflammatory diseases.

Abbreviations

UGT1A1: UDP-glucuronosyl transferase
HMOX: Heme oxygenase
HPRT: Hypoxantin phosphoribosyl transferase
ALT: Alanine aminotransferase
AST: Aspartate aminotransferase
ALP: Alkaline phosphatase
MDA: Malondialdehyde
IC: Inhibitory concentration.

Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

Disclosure

Part of the study has been presented at the 68th Annual Meeting of the American Association for the Study of Liver Diseases: The Liver Meeting, Washington, USA. The funding bodies had no role in the design of the study or collection, analysis, and interpretation of the data, or in writing of the manuscript.

Conflicts of Interest

The authors declare that they have no financial or commercial conflicts of interest.

Authors' Contributions

All authors contributed to the data analysis and interpretation and have read and approved the final manuscript.

Acknowledgments

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Supplementary Materials

Supplementary Material: a detailed analytical HPLC-MS method for the determination of silymarin flavonolignans together with obtained data from the analyses, i.e., detailed composition of silymarin complex used in the study. Supplementary Table 1: primers used for gene expression studies. *(Supplementary Materials)*

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Isolated flavonoids of silymarin complex increase systemic and hepatic concentrations of bilirubin and lower lipoperoxidation in mice

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HPLC and LC-MS analysis of silymarin

Experimental

The content of taxifolin and flavonolignans in silymarin (Sigma-Aldrich), and the calibration curves of taxifolin and silybin were measured on a Chromolith RP-18e (100 × 3 mm) column (Merck), Chromolith RP-18e (5 × 4.6 mm) precolumn (Merck), mobile phase: 2% acetonitrile, 37% methanol, 61% water, 0.1% HCOOH; isocratic elution; flow rate 1.1 mL/min, 25°C. The content of taxifolin in silymarin was calculated from the calibration curve of taxifolin; the content of silybin A, silybin B, silychristin A, silychristin B + silydianin (co-eluting), isosilybin A, and isosilybin B were determined using the calibration curve of silybin (possible differences in the extinction coefficients of the individual flavonolignans were neglected).

The content of 2,3-dehydroflavonolignans in silymarin and standard solutions of 2,3-dehydrosilybin was determined using LC-MS measured on a Chromolith RP-18e (100 × 3 mm) column (Merck), Chromolith RP-18e (5 × 4.6 mm) precolumn (Merck), mobile phase: A = 5% acetonitrile, 0.1% HCOOH; B = 80% acetonitrile, 0.1% HCOOH; gradient: 0 min 20% B, 5 min 90% B, 6 min 90% B, 8-10 min 20% B; flow rate 0.4 mL/min, 25°C. The MS parameters were as follows: ESI interface voltage, 4.5 kV; detector voltage, 1.15 kV; nebulizing gas flow, 1.5 $mL.min^{-1}$; drying gas flow, 15 $mL.min^{-1}$; heat block temperature, 200°C; DL temperature, 250°C; negative scan mode 478.8-481.0 *m/z*; software LabSolutions ver. 5.75 SP2 (Shimadzu, Kyoto, Japan). The contents of 2,3-dehydrosilybin, 2,3-dehydrosilychristin and at least two additional 2,3-dehydroflavonolignans were determined using the calibration curve of 2,3-dehydrosilybin.

Results

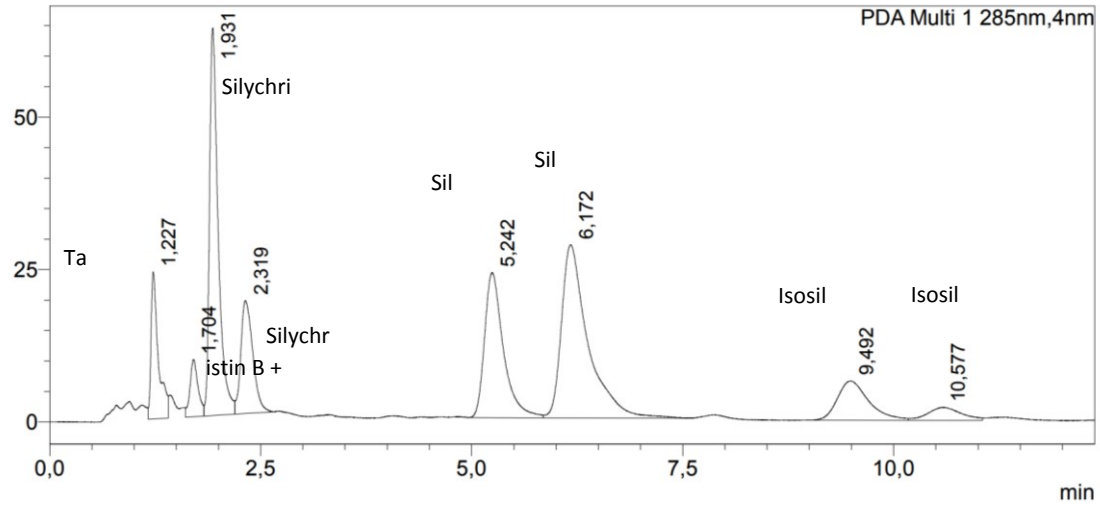
Physical properties, that is to say, extinction coefficients and retention times of 2,3-dehydroflavonolignans differ substantially from that of flavonols and flavonolignans; therefore, two separate methods had to be used for proper silymarin analysis. While taxifolin together with the flavonolignans could be identified through comparisons with standards and quantified on HPLC (Fig. S1), the 2,3-dehydroflavonolignans were determined by LC-MS (Fig. S2). Besides 2,3-dehydrosilybin and 2,3-dehydrosilychristin, which were identified using standards, at least two additional 2,3-dehydroflavonolignans having the same molecular mass and UV-vis spectra were identified in the LC-MS chromatogram, but did not correspond to any of the standards available (2,3-dehydrosilydianin, 2,3-dehydroisosilybin). After quantification of all identified compounds, 17.5% of silymarin content in the measured sample remained unknown and probably accounts for polymeric flavonolignans. Altogether, the silymarin sample used in the present study contained 13.0% of silybin A, 17.9% silybin B, 14.7% silychristin A, 9.3% silychristin B + silydianin, 8.9% isosilybin A, 6.8% isosilybin B, 3.0% taxifolin, 1.9% 2,3-dehydrosilybin, 0.5% 2,3-dehydrosilychristin, as well as 6.5% of other non-identified 2,3-dehydroflavonolignans and 17.5% of other substances, probably polymers (Fig. S3).

Suppl. Table 1. Primers used for gene expression studies.

HMOX 1
5′ GGGTGATAGAAGAGGCCAAGA-3′
5′ AGCTCCTGCAACTCCTCAAA-3′
UGT1A1
5′CAGAACTTTCTGTGCGACGTG-3′
5′GGGCCTAGGGTAATCCTTCAC-3′
HPRT **(reference gene)**
5′ CACTGGCAAAACAATGCAGAC 3 ′
5′ GGGTCCTTTTCACCAGCAAG 3 ′

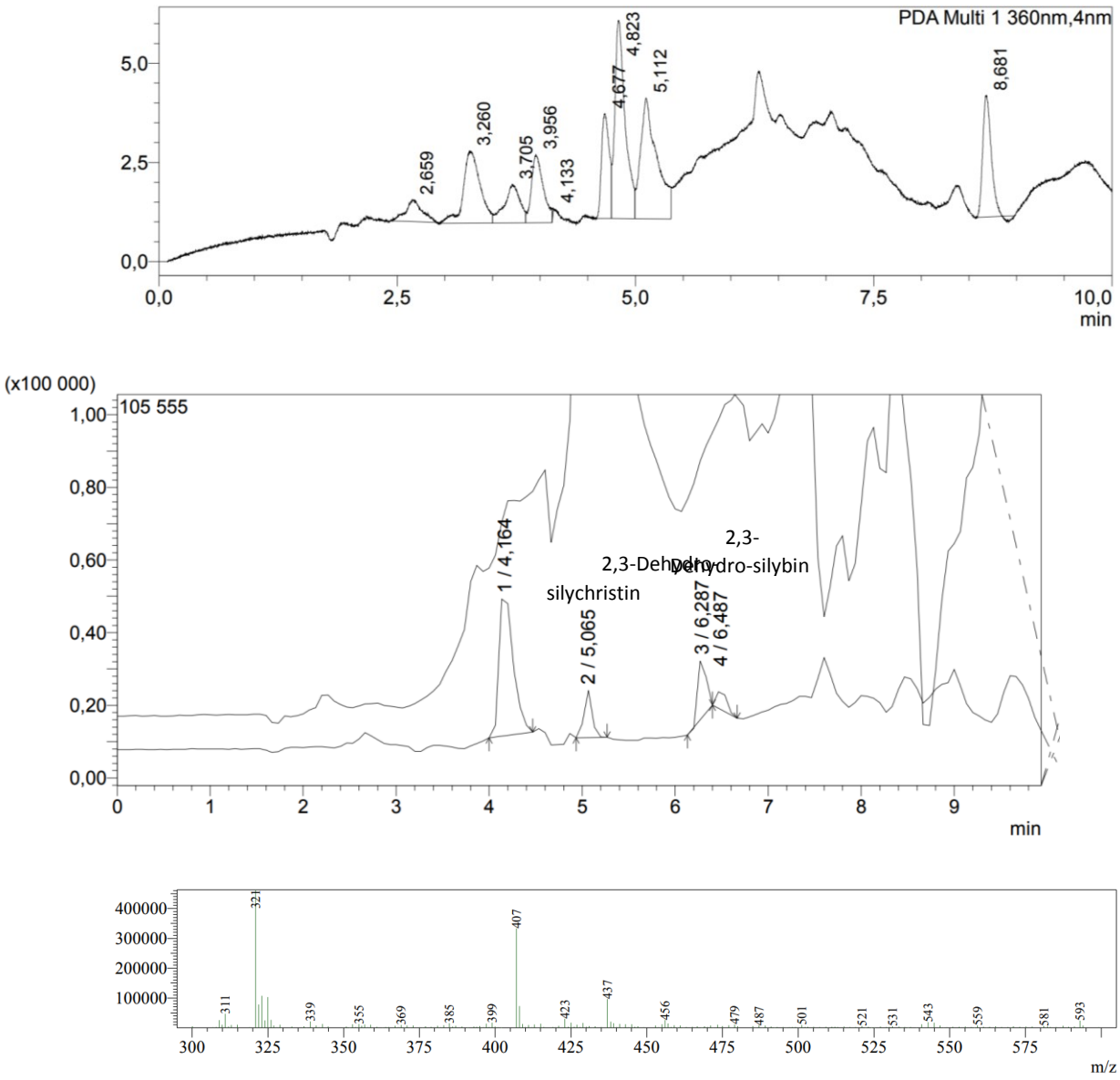
HMOX1, heme oxygenase 1; *UGT1A1*, bilirubin UDP-glucuronosyl transferase 1A1; *HPRT*, hypoxantin phosphoribosyl transferase.

Suppl. Fig. 1. HPLC chromatogram of flavonoids and flavonolignans in silymarin (Sigma) used.



Suppl. Fig. 2. LC-MS analysis of 2,3-dehydroflavonolignans in silymarin (Sigma) used in the study.

A: UV-Vis detection at 360 nm, **B**: single ion monitoring at *m/z* 479 (negative mode), **C**. representative MS spectrum



Suppl. Fig. 3. Content of individual flavonoids in silymarin sample (Sigma) used in this study.