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Katabolická dráha hemu v patogenezi jaterních onemocnění

Heme catabolic pathway in pathogenesis of liver diseases

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Disertační práce bude nejméně pět pracovních dnů před konáním obhajoby zveřejněna k nahlížení veřejnosti v tištěné podobě na Oddělení pro vědeckou činnost a zahraniční styky Děkanátu 1. lékařské fakulty.

Abstrakt

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

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

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

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

Abstract

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

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

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

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

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1 INTRODUCTION

1.1 Heme catabolic pathway

The heme catabolic pathway accounts for the degradation of heme, which is a term that in mammalian cells refers to an iron ion chelated in a porphyrin ring. The porphyrin ring of heme (ferroprotoporphyrin IX) is broken and oxidized at the α-methen bridge by heme oxygenase (HMOX, EC 1.14.99.3), producing equimolar amounts of carbon monoxide (CO), ferrous ion, and biliverdin that is immediately metabolized to bilirubin by biliverdin reductase (BLVRA, EC 1.3.1.24). This process requires a molecule of NADPH and oxygen [1].

To this date, two isoforms of HMOX have been described: an inducible form, heme oxygenase-1 (HMOX1) [2], and a constitutively expressed form, heme oxygenase-2 (HMOX2) [3]. HMOX1 is one of the most inducible genes which can be stimulated by many factors including heme, heavy metals, reactive oxygen species, curcumin, lipopolysaccharide, etc. (reviewed in [4]). The physiological importance of HMOX1 has been proved in HMOX1-deficient patient [5] and *hmox1*$^{-/-}$ mice [6] where the serious consequences of the heme degradation impairment can result in death.

The heme catabolic pathway serves not only to degradation of potentially pro-oxidant heme, but to provide several biologically active molecules. CO is, on the contrary to the hazardous impact of its intoxication, a ubiquitous and essential endogenous signaling molecule in mammalian system. It displays anti-inflammatory [7], anti-apoptotic [8], cytoprotective [9] and antiproliferative [10] effects. Furthermore, biliverdin/bilirubin acts as a strong antioxidant [11], anti-atherogenic [12], anti-inflammatory [13] and antiproliferative agent [14]. Last but not least, ferrous ion stimulates the synthesis of ferritin, which has been recognized for its antioxidant and anti-apoptotic properties [15].

1.2 Hepatic bile acids and bile pigment transport

1.2.1 Bile acids transport

Bile acids (BAs) represent a group of acid steroids with remarkable range of physical, chemical and biological effects in organism. BAs are synthesized from cholesterol, and their presence in organism is usually limited to the enterohepatic circulation, which includes the liver, bile ducts, intestine and portal blood [16].

Under normal physiological conditions, BAs are immediately transported to the canalicular membrane to be secreted to bile. The major transporters involved in mediating hepatic BAs secretion include bile salt export pump (*ABCB11*) and multidrug resistance-associated protein 2 (MRP2, *ABCC2*). The majority of both conjugated and unconjugated BAs are efficiently absorbed from the intestinal lumen back into blood. The unconjugated BAs can be rapidly transported into enterocytes via passive diffusion along the intestine whereas conjugated BAs require for absorption Na^+-dependent apical sodium dependent bile acid transporter. Subsequently, BAs are secreted through organic solute transporter α and β into portal blood. By transporting into hepatocyte via several transporters on the hepatocyte basolateral (sinusoidal) membrane, includig Na^+-dependent taurocholic cotransporting polypeptide (*SLC10A1*), microsomal epoxide hydrolase and organic anion transporting polypeptides (OATPs, *SLCO*), BAs enterohepatic circulation is completed (reviewed in [17]). However, during pathological conditions, BAs can be transported back to blood across the basolateral membrane by MRP3 (*ABCC3*) and MRP4 [18].

1.2.3 Bilirubin transport

Bilirubin released from heme degradation enters the blood circulation as unconjugated bilirubin (UCB) and circulates largely bound to albumin. The circulation of UCB in the bloodstream terminates after it reaches the hepatocyte and is transferred via basolateral membrane into the endoplasmic reticulum (ER). The exact mechanism of

UCB uptake is still unknown and both passive diffusion and active transport via several sinusoidal transporters have been proposed, including bilirubin/BSP-biding protein, organic anion-binding protein, bilitranslocase, and OATPs, namely OATP1B1 and OATP1B3. Inside the hepatocyte, UCB is transported within the cell bound to a group of cytosolic proteins, preferentially glutathione S-transferase B (ligandin or protein Y) and fatty acid-binding protein 1 (FABP1 or protein Z). Part of the UCB pool comprises of membrane-bound UCB. In ER, UCB is conjugated with glucuronic acid by bilirubin UDP-glucuronosyl transferase (UGT1A1, EC 2.4.1.17). The secretion of conjugated bilirubin into bile is mediated by MRP2 and ATP-binding cassette (ABC) efflux transporter (reviewed in [19]).

Interestingly, a significant fraction of conjugated bilirubin undergo "hepatocyte hopping" which means that conjugated bilirubin is secreted into the sinusoidal blood and subsequently re-uptaken via MRP3, OATP1B1 and OATP1B3 [20].

1.3 Pathogenesis of selected liver diseases

1.3.1 Cholestasis

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

The disruption in balanced bile salts metabolism is linked to various defects leading to the development of liver diseases, including cholestasis. Cholestasis may result from an impairment of bile formation, secretion or/and flow. The reduction in hepatic water and/or organic anion secretion (e.g. bilirubin, BAs) appears. These organic anions that are normally secreted into bile, are retained in blood. Cholestasis is typically characterized by an elevation in serum alkaline phosphatase activities, bilirubin and BA circulations, and if progressive, fibrosis, cirrhosis and clinical signs of liver failure may ultimately develop [21].

Cholestasis is classified as extra- and intrahepatic, and can be acute or chronic. Extrahepatic cholestasis is caused by an obstruction within the biliary tree. The most common causes of obstructive cholestasis are concrements in common bile duct or cancer of bile duct system and/or pancreas. In contrast, intrahepatic cholestasis is caused by hepatic transporter impairment, exposure to medicaments, sequels of hepatitis, hormonal imbalance, primary biliary cirrhosis or sepsis (reviewed in [21]). Estrogens were linked to cholestasis in susceptible women during pregnancy, administration of oral contraceptives or hormone replacement therapy [22]. Elevated levels of estrogens and progesterone in plasma in animals contribute to important changes in major hepatic transporters expression and regulation, representing the model of intrahepatic cholestasis in men [23].

The mechanism of hepatocellular damage in cholestasis is not clear but seems to be connected to the accumulation of toxic substances and subsequent free radicals production [24], disruption of the cytoskeleton and vesicle transport, impairment of signal transduction pathways, defects in tight junctional structures, and the destruction of bile ductules/ducts by immunological or toxic mechanisms [25].

1.3.2 Inflammation

Besides the metabolic and secretory function, the liver actively modulates inflammatory processes by filtrating, inactivating, and clearing bacteria, bacterial products, vasoactive substances, and inflammatory mediators [26]. Kupffer cells are the first to encounter bacterial lipopolysaccharide (LPS), in particular that originating from gut lumen. LPS in the bloodstream is bound to LPS binding protein forming a complex with a soluble or membrane-bound CD14 to bind to toll-like receptor 4 (TLR4) on the plasma membrane of cells in the liver. Activated TLR4 initiates the signaling pathway resulting in release of pro-inflammatory cytokines (TNFα, IL-1β, IL-6, IL-8,

chemokines) [27]. The release of pro-inflammatory cytokines triggers various cascades of inflammatory processes which, if not regulated, lead to liver injury.

In hepatocytes, inflammatory signals attenuate the expression of hepatocellular transporters both in the canalicular and basolateral membranes [28, 29]. Downregulation of hepatic transport systems affecting BA uptake and secretion, as well as downregulation of phase I and phase II detoxification systems, result in impairment in bile formation, and accumulation of BAs and toxins in the liver and serum [30]. Thus, inflammation-induced cholestasis becomes an integrated response to inflammatory signals of acute-phase response.

1.4 Heme catabolic pathway in liver diseases

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

The role of heme catabolic pathway in inflammation has been described, including direct actions in the liver by decrease of apoptosis [31, 32] and inhibiting cytokine production [31, 33]. Exogenous CO reduced the extent of apoptosis and pro-inflammatory stress response in the model of hepatic ischemia reperfusion injury [34].

Several studies suggest the role of CO in the maintenance of bile flow and liver integrity during cholestatic processes [35-37], including the direct effect on some hepatic transporters [38].

Collectively, these data demonstrate the importance of heme catabolic pathway in the protection of liver integrity via several mechanisms including anti-apoptotic [30], antiproliferative [31], vasoactive [35], anti-oxidative [11] and anti-inflammatory effects [7, 13]. However, the data on the role of the HMOX1 induction and its catabolic products in cholestatic disease and associated complications are lacking.

2 HYPOTHESIS AND AIMS

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

Hmox1 was previously shown to act as a hepatoprotective agent in endotoxin-induced liver injury. One of the Hmox1 inducers, curcumin, possesses anti-inflammatory and antioxidant properties. Whether curcumin exerts its inducing effects on Hmox1 under both physiological and stress conditions in the endotoxin/D-galactosamine model of liver injury has not been described yet. Thus, the aim of our study entitled „**Hepatoprotective effect of curcumin in lipopolysaccharide/D-galactosamine model of liver injury in rats: Relationship to HO-1/CO antioxidant system**" was to elucidate the effect of curcumin on Hmox1/CO antioxidant system under oxidative stress conditions in the liver [39].

As mentioned, Hmox1/CO system displays anti-inflammatory, antiproliferative, antioxidant and hepatoprotective properties. Moreover, CO has been shown as an important factor in maintaining the balance between liver sinusoidal perfusion and biliary transport, affecting bile canalicular contractility. In our study "**Protective effects of inhaled carbon monoxide in endotoxin-induced cholestasis is dependent on its kinetics**" we aimed to answer the question if this unique combination of anti-inflammatory and anticholestatic actions of CO might be useful in the treatment of endotoxin-induced cholestasis where the bile flow is impaired by inflammatory cytokines released by endotoxin. Since the capability of exogenously administered CO to reach target organs and its subsequent elimination ratio *in vivo* has not been evaluated so far, another aim of this study was to clarify whether inhaled carbon monoxide can reach the specific organs to exert its biological effects [40].

While BAs carry out several important functions in the organism affecting lipid metabolism, enzyme secretion, antimicrobial defense and nuclear factor targeting, its accumulation in hepatocytes during obstructive cholestasis is the major cause of

cholestatic liver injury resulting from oxidative stress. In our further studies we concentrated on the role of bilirubin that displays strong antioxidant properties. In study "**Bile acids decrease intracellular bilirubin levels in the cholestatic liver: implications for bile acid-mediated oxidative stress**" we aimed to elucidate the relation between cholestatic markers BAs and bilirubin regarding to their role in oxidative stress during cholestasis [41]. Since we have shown in the previous study that the intracellular unconjugated bilirubin can be depleted in oxidative stress-related diseases leading to the reduction of cell antioxidant capacity we aimed to explain intracellular metabolism and antioxidant properties of bilirubin under stress conditions *in vitro* as well as *in vivo* in the endotoxin-induced liver injury, and we reported results in „**Intracellular accumulation of bilirubin as a defense mechanism against increased oxidative stress**" [42].

Another cause of intrahepatic cholestasis are estrogens, which have been shown to alter hepatic transporter expression on both sinusoidal and canalicular membranes. Since the treatment of this condition is currently only symptomatic, we were interested if the *hmox1* induction can prevent estrogen-induced cholestasis in rats. Our results and effort to identify the possible mechanism of this regulation are reported in study "**Protective effect of heme oxygenase induction in ethinylestradiol-induced cholestasis**" [43].

3 MATERIALS AND METHODS

Animal experiments

All aspects of the animal studies met the accepted criteria for the care and experimental use of laboratory animals, and all protocols were approved by the Animal Research Committee of the 1st Faculty of Medicine, Charles University in Prague. In all experiments, rats were provided water and food ad libitum. All Wistar rats were obtained from Anlab (Prague, Czech Republic).

Bile duct ligation

Female Wistar rats and hyperbilirubinemic Gunn rats (RHA/jj, in-house colony from 1st Faculty of Medicine, Charles University in Prague) with a congenital deficiency of UGT1A1 were anaesthetized and microsurgical ligation of bile ducts and resections of extrahepatic biliary tracts were performed as previously described [44]. Sham-operated rats underwent the same procedure without bile duct resection and ligation. After 5 days, all animals were anaesthetized and blood from superior vena cava and the liver was collected.

LPS-induced liver injury and cholestasis

In first experiment, adult male Wistar rats were injected intraperitenoally (IP) with either 6 mg/kg LPS or with saline as a control. After 12 h, animals were anesthetized, blood was collected and the circulatory system was perfused with saline containing heparin to remove the blood. Liver, kidney, heart and brain were excised. In subsequent experiment, Wistar rats were injected IP with either 10 mg/kg of heme or saline (control) at day 1 and day 3. At the day 6, animals were anesthetized and livers and sera were collected.

In another study, female Wistar rats were divided into the 4 experimental groups: (1) control group, which received saline IP in the same volume as endotoxin-treated animals; (2) endotoxin-treated group, which received 6 mg/kg of LPS in saline IP; (3) CO-treated group which inhaled 250 ppm of CO (Linde Gas, Czech Republic) for 1 h; and received saline IP; and (4) CO- and LPS-treated group, 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. Organs (liver, heart, lung, kidney, spleen, brain, intestine, and muscles) and blood were collected.

Estrogen-induced cholestasis

Adult female Wistar rats were randomly divided into 4 groups: (1) those receiving only vehicle (propanediol); (2) those administered 5 mg/kg EE daily IP for 5 consecutive days; (3) those receiving 15 mmol/kg heme IP on days 0 and 3; and (4) those co-administered heme and EE at the above-mentioned doses or heme. On day 6, bile ducts were cannulated and bile was collected. In addition, all rats were cannulated with polyethylene tubes in the left carotid artery for blood sampling, and in bladder for urine collection. At the end of each experiment, animals were killed by exsanguination, and the livers were removed and weighed.

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 [45]. Hepatocytes were plated on collagen-coated cell culture dishes and maintained at 37°C, 5% CO2 in William's medium E, supplemented with penicillin/streptomycin, L-glutamine, insulin and 10% fetal 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. Cells were treated with vehicle, taurocholic acid TCA (10, 50, 100 μM), unconjugated bilirubin (25, 250 μM), EE (10 μM) and/or MHA (30 μM) 24 hrs after transfection.

Cell culture

The human hepatoblastoma cell line (HepG2) (purchased from American Type Culture Collection, Manassas, VA, USA) and HepG2 cell line stably transfected with Ntcp transporter (HepG2-rNtcp), kindly provided by Professor Ulrich Beuers (University of Munich, Germany; currently University of Amsterdam, Netherlands), were cultured as described previously [46].

HMOX activity measurement

Liver sonicate/cell suspension was incubated for 15 min at 37°C in CO-free septum-sealed vials containing methemalbumin (MHA) and NADPH as previously described [47]. Blank reaction vials contained 0.1 M phosphate buffer, pH 7.4, in place of NADPH. Reactions were terminated by adding 30% (w/v) sulfosalicylic acid. The amount of CO generated in the reaction and released into the vial headspace was quantitated by gas chromatography (GC) with a reduction gas analyser (Trace Analytical, Menlo Park, CA, USA). Hmox activity was calculated as pmol CO/hr/mg fresh weight (FW) of tissue.

Tissue CO measurement

Tissue sonicate was added to CO-free, septum-sealed vials containing 30% (w/v) sulfosalicylic acid. After 30 min incubation on ice, CO released into the vial headspace was quantitated by GC, as previously described [48]. CO content was calculated as pmol CO/mg FW.

Lipid peroxidation

Liver sonicate was incubated for 30 min at 37°C with ascorbate and Fe^{2+}. 2,6-di-tert-butyl-4methylphenol (BHT) was added for the blank reaction. CO produced into vial was quantitated by GC as previously described [49]. The amount of CO produced served as an index of lipid peroxidation and was expressed as pmol CO/hr/mg FW.

Carbonylhemoglobin (COHb) determination

CO in 1 mL of blood was measured by GC as described previously [50]. Total hemoglobin (tHb) was measured spectrophotometrically at 540 nm after the addition of 4 mL of whole blood to 2 mL of Drabkin's solution. The COHb was expressed as % tHb.

Real-time RT-PCR

Total liver RNA was isolated using phenol:chloroform extraction and cDNA was generated using random hexamer primers and Moloney Murine Leukemia Virus reverse transcriptase (Promega, Madison, WI, USA). Real-time PCR was performed with TaqMan® Gene Expression Assay Kit for *hmox1*. Data were normalized to glyceraldehyde 3-phosphate dehydrogenase and hypoxanthine phosphoribosyl transferase levels, and then expressed as fold change from control.

Alternatively, 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 (Life Technologies, Czech Republic) for *hmox1*, *IL10*, *IL6*, *TNF*, *Slc10a1*, *Abcc2, Abcc3*, *Slco1a1*, *Abcb11* (Life Technologies, Czech Republic). Data were normalized to β-2 microglobulin and expressed as fold change from control levels.

Western blots

One hundred micrograms of liver sonicates were mixed with equal volume of loading buffer. Samples were separated on 12% polyacrylamide gel and then transferred to polyvinylidene difluoride membranes (Millipore, Bedford, MA, USA). After blocking in Tween-PBS with 5% milk for at least 1 hr, membranes were incubated with Hmox1 antibody (1:666; Stressgen, Victoria, BC, USA), or β-actin (1:8000; Sigma-Aldrich) for 1 hr. After washing, membranes were incubated with anti-mouse IgG-HRP (1:2000; Sigma-Aldrich) for 30 min. After washing, immunocomplexes on the membranes were visualized with ECL Western Blotting Detection Reagents (Amersham Biosciences, Buckinghamshire, UK). Hmox1 protein bands were quantified by densitometry, normalized to β-actin, and then expressed as fold change from control.

Statistics

Normally distributed data were expressed either as mean ± SD [40, 41, 42, 43] or mean ± SEM [39], and non-normally distributed data as medians (25%-75%) [41, 43]. Normally distributed data were analyzed with Student *t*-test [39 - 43] and one-way ANOVA with *post hoc* Holm-Sidak test [40, 43] or Bonferroni test [39] for multiple comparisons. For non-normally distributed datasets, Mann-Whitney rank sum test [40, 41, 42, 43] and nonparametric Kruskal-Wallis ANOVA with Dunn's correction [40, 43] were used. The association between plasma bilirubin levels and antioxidant capacity was tested using Spearman rank-order correlation analysis [41]. *p*-Values less than 0.05 were considered statistically significant.

4 RESULTS AND DISCUSSION

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

In the first publication entitled „**Hepatoprotective effect of curcumin in lipopolysaccharide/D-galactosamine model of liver injury in rats: Relationship to HO-1/CO antioxidant system**“, we found that *hmox1* upregulation by curcumin pre-treatment protected the liver against damage induced by LPS/DG (which serves as an experimental model for an apoptotic acute liver failure). The hepatoprotection was clearly demonstrated by the significant decrease in serum aminotransferase activities (ALT, AST), decrease in lipid peroxidation measured as the production of conjugated dienes, nitrate production and total liver CO level and downregulation of *NOS2* gene expression after curcumin pre-treatment. Curcumin pre-treatment in the LPS/DG group was accompanied by significant increases in Hmox activity, *hmox1* expression and serum bilirubin levels contributing significantly to the hepatoprotective effect of

curcumin. We concluded the curcumin effect on the NOS-2/NO system downregulation and decrease in accumulation of lipid peroxidation products *in vivo* is mediated via the Hmox system [39].

In our other study, we used the model of LPS-induced liver injury to answer the question if the modulation of heme oxygenase pathway could specifically alter the cholestatic effects of LPS. The results are recorded in the study entitled „**Protective effects of inhaled carbon monoxide in endotoxin-induced cholestasis is dependent on its kinetics**“. In accordance with our previous results on the protective effects of Hmox activation in LPS-induced liver injury we found that CO pre-treatment before LPS administration significantly decreased hepatocellular injury, as evident by decrease of AST activities, as well as markers of cholestasis (ALP, BAs) indicating that CO possesses important hepatoprotective properties. To further clarify the mechanism of this effect, we were interested in selected inflammatory cytokines and if their modulation can affect the expression of hepatic transporters. We found that CO pre-treatment modulated the expression of inflammatory cytokines preventing the downregulation of hepatic transporters in time-dependent manner. The major effect of CO on the expression of these transporters was observed within 1 h of CO administration when the actual concentration of CO was still significantly increased. As we could not answer if this early transient protective effect on hepatic transporter expression followed by a later decrease in serum cholestatis markers can protect from long-term liver damage or disease development, further studies could clarify this question. In conclusion, we demonstrated that CO exposure substantially attenuated endotoxin-induced cholestasis in the manner, which might be connected to inflammatory cytokines and subsequent changes in hepatic transporters expression. This effect seems to be directly related to the kinetics of inhaled CO that has not been previously described. Thus in the same study, we characterized the kinetic profile of inhaled CO for the first time. We established the detailed CO elimination profiles for different organs and kinetic parameters for the rat. We found that both CO concentrations and

elimination half-lives were tissue dependent with biphasic elimination curves in all studied tissues. These data indicate the necessity of carefully designing studies with CO application [40].

The role of bilirubin in cholestatic diseases was investigated in the study entitled „**Bile acids decrease intracellular bilirubin levels in the cholestatic liver: implications for bile acid-mediated oxidative stress**“. While we found elevated markers of cholestasis (BAs, ALP and bilirubin) after bile duct ligation we simultaneously measured increase in the anti-oxidative capacity of plasma that positively correlated with bilirubin levels. In this model of extrahepatic cholestasis we showed for the first time that intracellular bilirubin is consumed during cholestasis and that plasma bilirubin levels might not reflect tissue bilirubin metabolism. In the course of investigating the mechanism of bilirubin decrease in the liver we found the increase in lipid peroxidation. Moreover, taurocholic acid (TCA) downregulated the expression and activity of Hmox increasing lipid peroxidation *in vitro*, which was abolished by adding bilirubin to the homogenate. In conclusion, even though bilirubin is an antioxidant, its concentration in the cholestatic liver can be depleted by high concentration of BAs [41].

We further extended our research on protective effects of UCB in human hepatoblastoma cells (HepG2) and rat liver cells exposed to other oxidative stressors and LPS, with focus on possible mechanisms responsible for the regulation of intracellular UCB levels. We concluded our results in the paper entitled „**Intracellular accumulation of bilirubin as a defense mechanism against increased oxidative stress**“. We found that heme-mediated *HMOX1* induction in HepG2 cells led to an elevation in intracellular UCB, which was associated with a dramatic decrease in lipid peroxidation. Furthermore, we measured lower accumulation of UCB and higher degree of lipid peroxidation in the liver of rats with LPS-induced cholestasis. The treatment of HepG2 with sodium azide or TCA led to a dramatic depletion of intracellular UCB. This depleting process was completely abolished by increased production of UCB by HMOX

or attenuated under hyperbilirubinemic conditions. To conclude, the balance between intracellular and extracellular UCB levels can be disturbed by different oxidative stimuli [42].

As products of the heme catabolic pathway showed an evident protective role in obstructive and sepsis-induced cholestasis we further focused on studying the role of *hmox1* induction in ethinylestradiol (EE)-induced cholestasis, representing the intrahepatic cholestasis of pregnancy. In study entitled „**Protective effect of heme oxygenase induction in ethinylestradiol-induced cholestasis**" we found that *hmox* induction by heme increased hepatocyte transporters expression and subsequently stimulated bile flow in cholestasis resulting in normal plasma BAs levels, and thus could confer to protection against EE-induced cholestasis. While TCA was found to be a potent inhibitor, prolonged treatment with EE caused significant increase in Hmox activity. In our study, heme treatment significantly decreased serum cholestatic markers and increased biliary output of BA and glutathione in EE-treated animals. Heme treatment partly prevented the downregulatory effect of EE on hepatic transporters and regulated the renal clearance and re-absorbtion transporters securing the bile flow in cholestatic rats. We confirmed on primary rat hepatocytes that Nrf2 is the key target in heme-mediated *Abcc3* overexpression, which leads to the effective clearance of BA into plasma and subsequently by the kidneys to urine [43].

5 CONCLUSIONS

While heme seems to be a controversial compound, usually presented as a deleterious pro-oxidative molecule, otherwise showing beneficial effects in several diseases, the heme catabolic pathway is largely connected to the protection of cells from harmful insults. Despite the original thought that the products of heme catabolic pathway are only toxic waste products, the beneficial and signaling actions have been extensively documented. CO displays anti-inflammatory, antioxidant and vasoactive

actions, while bilirubin is strong antioxidant with antiproliferative and anti-inflammatory effects.

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

As bilirubin is known for its anti-oxidative effects we investigated its role in the model of obstructive cholestasis that is connected to oxidative injury caused largely by accumulation of BAs. The experiments revealed that the level of intracellular bilirubin was very important in protecting the liver from oxidative damage and was not reflected by total plasma bilirubin level. Evoking hyperbilirubinemic conditions either by bilirubin supplementation or *hmox1* upregulation led to important hepatoprotection.

Furthermore, we described Hmox activity in liver tissue under cholestatic conditions caused by an administration of estrogens, which is the model of intrahepatic cholestasis of pregnancy. We discovered that Hmox activation by heme regulated the expression of renal and hepatic transporters resulting in alternative BAs clearance into plasma and subsequently to urine by the kidneys.

To conclude, we discovered important involvement of heme and HMOX in cholestatic and inflammatory pathways in the liver. Our results suggest not only an important hepatoprotective role of the products of heme catabolic pathway, but reflect

the importance of maintaining the level of intracellular bilirubin which might represent an important pharmacological target. The regulation of HMOX and UGT1A1 activities might represent the therapeutical strategy for treatment not only cholestatic disorders, but various diseases connected to oxidative stress and inflammatory processes.

6 LIST OF ABREVIATIONS

Abcb11	gene for bile salts export pump
Abcc2	gene for MRP2
Abcc3	gene for MRP3
ALP	alkaline phosphatase
ALT	alanine aminotransferase
AST	aspartate aminotransferase
BAs	bile acids
BLVRA	biliverdin reductase
CO	carbon monoxide
COHb	carbonylhemoglobin
DG	D-galactosamine
EE	ethinylestradiol
ER	endoplasmic reticulum
HMOX/Hmox	heme oxygenase human/rat, mice
IL	interleukin
LPS	lipopolysaccharide
MHA	methemalbumin
MRP/Mrp	multidrug resistance-associated protein
NADPH	nicotinamide adenine dinucleotide phosphate
NOS2	nitric oxide synthase 2
Nrf2	nuclear factor erythroid 2-related factor-2
NTCP/Ntcp	Na+-taurocholate cotransporting protein
OATP/Oatp	organic anion transporting polypeptide
Slco	gene for Oatp transporter
Slc10a1	gene for Ntcp transporter
TCA	taurocholic acid
TLR	toll-like receptor
TNF	tumor necrosis factor
UCB	unconjugated bilirubin
UGT1A1	bilirubin UDP-glucuronosyl transferase

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