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Autoreferát disertační práce

UNIVERZITA KARLOVA
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Indukce hemoxygenasy a biologická úloha jejích metabolických produktů.

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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

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, an 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 oxygenase and its products

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 (Tenhunen R. et al., 1972). HMOX cleaves heme molecules to yield equimolar quantities of biliverdin IXa, CO, and free iron. Biliverdin is subsequently converted to bilirubin via the action of biliverdin reductase, and free iron is promptly sequestered into ferritin (Otterbein L.E. & Choi A.M.K., 2000). 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).

The importance of HMOX is well documented in cases of HMOX1-deficiency ($HMOX1^{-/-}$) in mice and humans. Patients 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).

Heme degradation pathway forms two biologically active products, bilirubin and CO.

CO is a colorless, odorless gas, mostly know as a product of incomplete combustion of organic compounds, and it is highly dangerous for its high affinity to hemoglobin followed by tissue hypoxia (Ryter S.W. & Otterbein L.E., 2004). Since 1993 when CO role as a neurotransmitter was discovered, its beneficial role has been intensively studied (Verma A. et al., 1993). CO has shown its protective properties *in vivo* especially in a therapy of systemic inflammation (Knauert M. et al., 2013), liver and intestine inflammatory diseases (Šuk J. & Muchova L., 2018), cancer treatment (Szabo C., 2016) or I/R injury and organ transplant (Ozaki K.S. et al., 2012).

Bilirubin, the end product of the heme catabolic pathway, has long been recognized as a marker of liver dysfunction or a potential toxic compound causing severe brain damage in newborns. But unconjugated bilirubin (UCB) is also an important antioxidant that represents 10% of blood antioxidant capacity (Belanger S. et al., 1997). Mildly elevated bilirubin levels in serum were associated with a significantly lower prevalence of colorectal cancer and a reduction in cardiovascular disease risk (Schwertner H.A. & Vitek L., 2008).

UCB has also anti-inflammatory and cytoprotective properties (Gazzin S. et al., 2016). 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), proteinkinase C (PKC) (Amit Y. & Boneh A., 1993) and many others.

1.2 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 conjugated bilirubin (CB) being a negligible part (Gazzin S. et al., 2017). While mildly elevated systemic bilirubin levels, are believed to have protective effects against oxidative stress related diseases, highly elevated levels (above 340 μM) presumably in neonates could be associated with bilirubin destructive effects like kernicterus or bilirubin-induced neurological dysfunction (Gazzin S. et al., 2016).
Hyperbilirubinemia can be divided into three different categories including pre-hepatic, intra-hepatic, or post-hepatic (Beckingham I.J. & Ryder S.D., 2001).

1.2.1 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 elevation of UCB typically seen in Gilbert syndrome patients 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 (Novotny L. & Vitek L., 2003; Vitek L. et al., 2002).

1.3 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.

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 the role of HMOX induction by heme in prevention of ethinylestradiol 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 has an 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 MATERIAL AND METHODS

Following list represents methods used in the submitted dissertation thesis by the author. Detail description and other information about particular method are listed in publications related to this thesis in sections “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 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 a product of heme degradation pathway and a 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 (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). Induction of HMOX in ethinylestradiol (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 (Mrp2) transporters, where their repression caused by EE treatment was reversed by HMOX induction. The only transporter specifically activated by heme was Mrp3. The HMOX induction by heme and Mrp3 regulation is mediated via Nrf2 pathway. 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. 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 on 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.

As we show in the study and as a 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 pro-inflammatory cytokines including tumor necrosis factor α (TNFα), interleukin-1β (IL-1β) and interleukin-6 (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 *IL-10* 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. Phosphatidylinositol-3 kinase (Akt) pathway seems to play an 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. 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).

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., 2016). 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 (Zelenka J. et al., 2012), or decreasing its elimination by inhibition of bilirubin UDP-glucuronosyl transferase (UGT1A1) (Dekker D. et al., 2011).

Our *in vitro* results show that flavonolignans of silymarin inhibit *UGT1A1* mRNA expression and also increase intracellular concentration of UCB *in vitro* among those dehydrosilybin was the most potent flavonolignan.

In our study, we have shown that dehydrosilybin affects bilirubin pathway and is even better inhibitor of UGT1A1 than silybin, largely studied silymarin constituent and known UGTs inhibitor.

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.

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). 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.

5 CONCLUSIONS

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 transporters 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.

6 LIST OF ABERRATION

Ahr - arylhydrocarbon receptor

Akt – protein kinase B

BV – Biliverdin

BVR – biliverdin reductase

CB - conjugated bilirubin

cGMP – cyclic guanosine monophosphate

CO – carbon monoxide

COHb – carbonyl hemoglobin

CORM - carbon monoxide releasing molecule

EE – Ethinylestradiol

GTP - guanosine triphosphate

HMOX - Heme oxygenase

LC/MS/MS – Liquid chromatography tandem mass spectrometry

LCAT – Lecithin–cholesterol acyltransferase

LPS - Lipopolysaccharide

Mrp – Multidrug resistance-associated protein

NADPH - Nicotinamide adenine dinucleotide phosphate

NOS – nitric oxide synthase

Nrf2 - nuclear factor (erythroid-derived2)-like2

NTCP – sodium/taurocholate cotransporting polypeptide

OATP - organic anion transporting polypeptides

PKC - proteinkinase C

RNAi - RNA interference

sGC - soluble guanylate cyclase

shRNA - short harpin RNA

siRNA - short interfering RNA

UCB – unconjugated bilirubin

UGT1A1 - UDP- glucuronosyl transferase 1A1 isoform

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