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8. List of publications

Publications related to thesis:

1. Vitek L., Muchova L., Zelenka J., Zadinova M., Malina J. The Effect of Zinc Salts on Serum Bilirubin Levels in Hyperbilirubinemic Rats. J. Pediatr. Gastroenterol. Nutr. 2005;40:135–140. IF 1.8

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Publications with different objectives

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Enterohepatic circulation of bilirubin

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unconjugated hyperbilirubinemia which occurs within one week after delivery in more than 90% of newborns. It results predominantly from high rate of hemoglobin degradation and unsufficient conjugating capacity of the newborn liver. In addition neonatal intestine is poorly colonised with bilirubin reducing microflora and maternal milk usually possesses high activity of β-glucuronidase [3]. Thus, newborn colonic UCB levels are much higher than in adults forming gradient enhancing EHC and ESC which cumulatively increase the level of hyperbilirubinemia [4].

The pigment gallstones are formed mainly by precipitation of calcium bilirubinate in the bile. Development of such a concretion requires presence of high concentration of UCB in a bile duct. EHC could significantly increase biliary bilirubin levels and prefere formation of more labile bilirubin monoglucuronoside instead of bilirubin bisglucuronoside due to saturation of conjugation enzymes. This phenomenon is particularly enhanced in Gilbert syndrome patients and patients with bile salt malabsoption.

Level of free intestinal UCB is the main driving force for EHC. There are numerous agents causing intestinal UCB precipitation or strong adsorption thus inhibiting EHC and ESC. Adsorbents like agar and cholestyramine or precipitation agents like calcium phosphate and zinc sulphate were succesfully tested in animal models. However, therapeutic value of these substances for treatment of neonatal jaundice or pigment gallstone formation is discutable due to possible adverse effects and inconsistent results from clinical studies.

2. Aims

Inhibition of bilirubin EHC by agents decreasing its intestinal solubility is a promising approach in treatment of neonatal jaundice. However, adsorbents and precipitation agents used so far were not clinically applicable. In our first paper **„The effect of zinc salts on serum bilirubin levels in hyperbilirubinemic rats“**, we aimed to investigate hypobilirubinemic effect of zinc methacrylate - the mixed effect adsoption/precipitation agent.

Our knowledges about role of intestinal microflora in bilirubin EHC are rather scarce. We isolated several strains of bacteria from neonatal stool and characterized their bilirubin reducing activity. The aim of our next paper **„The impact of intestinal microflora on serum bilirubin levels“** was to investigate their influence on serum UCB levels in Gunn rats.

The intestinal metabolic pathway of BP is known only in context of whole intestine. Metabolism of particular strain of bilirubin reducing bacteria was never investigated. The aim of our third study **„Identification of bilirubin reduction products formed by *Clostridium perfringens* isolated from human neonatal fecal flora“** was to investigate BP metabolism in sole strain of *Clostridium perfringens* with high rate of urobilinoid production.

Metabolism of bilirubin under physiological and pathological conditions was investigated only indirectly using determination of bilirubin species in serum, urine or bile. However, knowledges about tissue and cellular UCB levels are

principial for our further understanding of bilirubin metabolism. Unfortunately, tissue UCB levels are very low and it is a very labile substance with high affinity to biomolecules. For these reasons, there was no analytical procedure for sensitive determination of bilirubin in complex biological matrices. In our ultimate paper **„Highly sensitive method for quantitative determination of bilirubin in biological fluids and tissues“**, we aimed to develop such a method.

transported from periphery to the liver bound on serum albumin. Hepatocytes conjugate UCB with glucuronic acid using enzyme bilirubin:UDP glucuronosyltransferase. Bilirubin conjugates are then exported into bile. Insufficient activity or complete missing of the enzyme function lead to severe indirect hyperbilirubinemia, and on condition that the blood-brain barrier is compromised, e.g. during neonatal period, result in neurological damage called kernicterus [3].

Conjugated bilirubin is excreted into bile via active transporter multidrug resistance-associated protein-2 (MRP2) which serves as a canalicular exporter for various conjugated xenobiotics and endogenous compounds. During passage through small intestine and colon, most of bilirubin is deconjugated. Part of the intestinal UCB is reabsorbed and transported via portal vein to the liver and from larger part also to the systemic circulation. This process called enterohepatic and enterosystemic circulation (EHC, ESC), respectively is influenced by the ratio between bilirubin deconjugation and transformation to other compounds and by the relative solubility of UCB in the intestinal content. However, under physiological conditions, the vast majority of UCB remains in the intestine and is hydrogenated by intestinal microflora to urobilinoids. Particular strains of *Clostridium perfringens* and *C. difficile* capable to reduce bilirubin were identified in our laboratory [4].

EHC and ESC of bilirubin are important components in the pathogenesis of neonatal jaundice as well as pigment gallstones formation during bile acid malabsorption and Gilbert syndrome. Neonatal jaundice is a potentially brain damaging

1. Introduction

Bile pigments (BP) are group of substances descending from physiological degradation of heme and bearing common structural motif of linear tetrapyrrole. Number of BP with various physiological functions was described to be produced by animals, plants and procaryotic microoorganisms [1]. Human BP metabolic pathway is opened by microsomal enzyme Heme oxygenase which oxidatively cleaves heme moiety producing biliverdin, carbon monoxide and free iron. Biliverdin, the blue-green polar substance, is present only in trace amounts in human body. It is readily reduced by enzyme biliverdin reductase (BVR) forming major mammalian bile pigment bilirubin. BVR is a microsomal and cell surface enzyme playing an important role in cellular oxidative stress signalling.

Unconjugated bilirubin (UCB), the orange nonpolar substance, possess important antioxidant and antiinflammatory properties in physiological and mildly elevated range of concentrations [2] while it could be neurotoxic under pathological conditions [3]. UCB is potent antioxidant and protects serum albumin as well as LDL particles against various free redical species. I addition, number of clinical studies have shown that subjects with Gilbert syndrome are significantly protected against atherosclerosis and colorectal cancer [2]. Pathogenesis of all these disorders was found to be accompanied with oxidative stress and chronic inflammation. UCB is produced as a result of continuous degradation of hemoproteins including hemoglobin from senescent red blood cells in spleen and is

3. Methods

Animals: Adult Wistar rats or Gunn rats (250-270 g) fed with standard diet and treated according criteria for the human care and experimental use of laboratory animals approved by the Animal Research Committee of the 1st Faculty of Medicine were used in all the studies.

Bacteria: Strain of *Clostridium perfringens* with high bilirubin reducing capacity was cultured under anaerobic conditions in medium containing phosphate buffer and yeast broth without shaking. To determine bilirubin reducing capacity, bacteria were cultured overnight in medium containing UCB and urobilinoids were determined spectrophotometrically.

Analyses of urobilinoids: Urobilinogen was determined spectrophotometrically as a complex with Zn ions after oxidation to urobilin. Other pigments were analysed using thin layer chromatography on silicagel in chloroform/methanol phase and their chromatographic properties were compared with those of known standards.

HPLC determination of UCB: Tissues were homogenised, internal standard was added and UCB extracted into chloroform. Then, another extraction into minute volume of alkaline aqueous solution was performed and resulting droplet was injected onto HPLC column. Separation was performed in water/methanol phase on C8 reverse phase column with detection at 440 nm.

4. Results and Discussion

In the presented papers, important components of bilirubin EHC were characterized in rodent models. Generally, novel basic approaches for regulation of neonatal jaundice were explored in the first two publications while the other two clearly characterized the previously confounded BP metabolism in the bacterial cells and mammalian tissues, respectively.

In the first publication „**The effect of zinc salts on serum bilirubin levels in hyperbilirubinemic rats**“, important decrease in serum bilirubin levels was found in response to feeding animals with insoluble UCB binding agent zinc methacrylate. However, the relevance of zinc methacrylate for future treatment of severe unconjugated hyperbilirubinemia is highly discutable. Although we found no pathologic changes in the rat intestine at the end of the experiment, methacrylate should be considered as a toxic agent. However, the important conclusion from this study is, that intestinal passage of unabsorbable and inert material with high ability to specifically adsorb UCB (e.g. using divalent cationts) can significantly decrease level of unconjugated hyperbilirubinemia due to its ability to restrict EHC and ESC of bilirubin. For treatment of neonatal jaundice, biocompatible polymers without any chemical interaction with organism can be developed in future.

Another approach for restriction of EHC and ESC of bilirubin is enhancement of bilirubin reduction. This is highly suitable especially for treatment of newborns lacking effective intestinal microbial ecosystem. In the second paper „**The impact**

found to be a main product of hydrogenation of a number of substrates including mesobilirubin, bilirubin dimethylester, bilirubin ditaurate but not bilirubin bisglucuronoside.

Also the investigation of cellular and tissue bilirubin metabolism is important for understanding of its EHC as well as its antioxidant and toxic properties. However, due to its low cellular levels and high instability, valid analytical method for tissue bilirubin determination was unavailable. Therefore, we developed and validated a highly sensitive and precise HPLC assay for quantification of bilirubin in cells and tissues under normobilirubinemic as well as hyperbilirubinemic conditions.

Souhrn

Bilirubin, hlavní produkt degradace hemu, působí fyziologicky jako antioxidant, zatímco ve vysokých koncentracích může být neurotoxický. Z těla je vylučován jako součást žluči do střeva, kde zůstává vázán na střevní obsah a je postupně metabolizován střevní mikroflórou. Část bilirubinu je resorbována zpět do oběhu a prochází tzv. enterohepatální cirkulací (EHC). Náplní této disertační práce je studium metabolismu a EHC bilirubinu.

Bilirubin se ve střevě váže s dvoumocnými kationty a tím je utlumena jeho EHC. Nicméně případné terapeutické podávání takových látek může ovlivnit metabolismus anorganických iontů v těle. Výsledky naší první studie ukazují, že pokud byly Gunnovy krysy s vrozenou hyperbilirubinémií krmeny nerozpustným metakrylanem zinečnatým, jejich hladiny sérového bilirubinu poklesly, zatímco koncentrace zinku v krvi zůstala nezměněna a zároveň se neobjevily žádné patologické změny trávicího traktu.

EHC bilirubinu je významně ovlivněna také rychlostí redukce bilirubinu střevní mikroflórou, především bakteriemi rodu *Clostridium*. V naší druhé studii jsme na Gunnových krysách prokázali, že antibiotická terapie vedoucí k eliminaci střevních klostridií vedla k významnému vzrůstu hyperbilirubinémie, zatímco následná kolonizace kmenem *C. perfringens* s vysokou aktivitou enzymů redukujících bilirubin vedla k významnému poklesu sérového bilirubinu směrem k původním hodnotám.

5. Conclusions

a) Oral treatment with unabsorbable matrices bearing high bilirubin binding capacity is a promising approach in treatment of neonatal hyperbilirubinemia and/or Crigler-Najjar syndrome.

b) Bilirubin reducing activity of intestinal bacteria strongly influences level of hyperbilirubinemia in Gunn rats suggesting that probiotic bacteria capable to reduce bilirubin might be the future treatment for neonatal jaundice.

c) Bacterial reduction of bilirubin to urobilinoids is a complex multi-step procedure requiring simultaneous presence of hydrolases, membrane transporters and reducing enzymes. Thus further investigation is needed.

d) A novel HPLC based method for determination of bilirubin in tissues was developed and validated allowing direct investigation of tissue and cellular bilirubin metabolism during various physiological as well as pathological events.

6. Abbreviation

BP, bile pigment; EHC, enterohepatic circulation, ESC, enterosystemic circulation; HO-1, heme oxygenase-1; UCB, unconjugated bilirubin;

7. References

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Summary

Bilirubin is a main physiological product of heme degradation possessing antioxidant properties at low concentrations as well as neurotoxicity under pathological conditions. It is secreted from the body into bile and is further metabolised in the intestine. It could be either reduced to urobilinoids, adsorbed on the intestinal content or reabsorbed by the intestinal wall and take a part in enterohepatic or even enterosystemic circulation of bilirubin (EHC). In the presented thesis, the mechanisms affecting EHC of bilirubin and tools for further research in bilirubin metabolism were investigated.

EHC of bilirubin could be decreased by precipitation with divalent cations in the intestine. However, therapeutic feeding with such salts could significantly influence homestasis of inorganic ions. Our study proved that feeding of hyperbilirubinemic Gunn rats with insoluble salt zinc methacrylate led to important decrease in serum bilirubin levels of hyperbilirubinemic Gunn rats without affection of zinc metabolism and pathologic changes in the intestine.

Another way to decrease EHC is reduction of bilirubin by intestinal bacteria belonging to the genus *Clostridium*. We proved that eradication of intestinal *Clostridia* in Gunn rats led to significant increase in hyperbilirubinemia while recolonization with a sole strain of *C. perfringens* capable of reducing bilirubin partially restored the bilirubin homeostasis.

Bilirubin metabolism of this bacterial strain was further characterized using chromatographic methods. Urobilinogen was

of intestinal microflora on serum bilirubin levels“, we proved that eradication of UCB reducing microflora had led to increase in serum bilirubin levels in Gunn rats, while recolonization with single strain possessing bilirubin reducing capacity led to important drop of hyperbilirubinemia. However, bilirubin reducing capacity is associated with potentially pathogenic strains of *Clostridium perfringens* and *C. difficile* which application as a probiotic agent for newborns is highly unlikely. The solution is preparation of safe transgenic bacterial strain with enhanced UCB reducing activity. For this reason, further characterization of metabolic pathway leading to urobilinoids is highly needed.

Initially, production of urobilinoids was characterized in strain of *C. perfringens* isolated from neonatal stool. In the third publication „**Identification of bilirubin reduction products formed by *Clostridium perfringens* isolated from human neonatal fecal flora**“, unconjugated urobilinogen was found as a main physiological product of UCB reduction in this bacteria. Moreover, production of urobilinoids was also possible from UCB derivatives mesobilirubin, bilirubin dimethylester, bilirubin bisglucuronoside and much less effective also from bilirubin ditaurate and bilirubin diethylester. Bilirubin bisglucuronoside was found to be deconjugated prior to reduction. Further characterization, isolation and finally identification of DNA sequence encoding genes for bilirubin-reducing enzymes is needed for potential preparation of possible transgenic probiotic bacteria.

Not only the end of BP metabolic pathway but also the beginning in mammalian cells and tissues is still not yet fully recognised. This is also due to difficulties in BP analysis. Since UCB is highly sensitive to oxidation and possess high affinity to biomolecules, there has been no reliable method for determination of low physiological UCB levels in cells and tissues. In our fourth publication „**Highly sensitive method for quantitative determination of bilirubin in biological fluids and tissues**“, we developed such a highly sensitive and precise method and used it to determine bilirubin levels in tissues from Wistar and Gunn rats. The novel method is suitable tool for investigation of UCB cytotoxicity, detoxication mechanisms and active transport together with correlation of heme oxygenase activity with production of bilirubin, study of changes in UCB levels in response to oxidative stress and possible antiinflammatory and cytostatic function of UCB. The knowledge of these processes together with deeper knowledge of bilirubin EHC and intestinal metabolism will help to treat neonatal jaundice and, on the other hand, regulate UCB levels to enhance its beneficial antioxidant and antiinflammatory action.

V další práci byl pomocí chromatografických metod charakterizován bilirubinový metabolismus výše zmíněného kmene. Bakterie byly schopny redukovat celou řadu substrátů včetně mesobilirubinu, dimethylesteru bilirubinu a bilirubin ditaurátu. Bilirubin bisglukuronosid musel být před redukcí dekonjugován. Produktem redukce byl nekonjugovaný urobilinogen.

V poslední práci jsme se zaměřili na vývoj a validaci analytické metody vhodné pro stanovení fyziologických množství bilirubinu ve tkáních a tkáňových kulturách. Přestože současné výzkumy připisují bilirubinu významnou roli v buněčných pochodech, dosud neexistovala vhodná metoda na jeho stanovení, především kvůli nízkým koncentracím ve tkáních, afinitě k biomolekulám a malé stabilitě. Podařilo se nám vyvinou vysoce senzitivní a specifickou HPLC metodu a stanovit koncentrace bilirubinu ve všech zkoumaných tkáních z normobilirubinemických i hyperbilirubinemických krys.