**UNIVERZITA KARLOVA V PRAZE**

Farmaceutická fakulta v Hradci Králové

Katedra farmakologie a toxikologie

# Vliv modulace zánětu na exkreční mechanismy během intrahepatání cholestázy

Dizertační práce

Školitel: Prof. PharmDr. František Štaud, PhD.

Konzultant: Prof. MUDr. Stanislav Mičuda, PhD.

Hradec Králové 2017 Mgr. Zuzana Kadová

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V Hradci Králové 21.4. 2017 ...……..

Mgr. Zuzana Kadová

## Poděkování

Tímto bych chtěla poděkovat svému školiteli Prof. PharmDr. Františku Štaudovi, PhD. za to, že mi umožnil postgraduální studium na katedře farmakologie. Můj největší dík patří hlavně mému konzultantovi Prof. MUDr. Stanislavu Mičudovi, PhD., bez jehož podpory, trpělivosti a odborných připomínek bych se při postgraduálním studiu a psaní dizertační práce neobešla. Dále děkuji všem spolupracovníkům z katedry farmakologie na lékařské fakultě za jejich vstřícnost, spolupráci a praktickou pomoc. Děkuji i mé rodině za podporu nejen materiální, ale i motivační.

# Abstrakt

Univerzita Karlova v Praze

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**Název dizertační práce:**

## Vliv modulace zánětu na exkreční mechanismy během intrahepatální cholestázy

Intrahepatální cholestáza doprovází celou řadu onemocnění a může být vyvolána i některými léčivy. Všechny formy cholestázy jsou spojeny s určitým stupněm zánětu. Cíl této studie byl tedy zaměřen na hodnocení změn v jaterní a ledvinné eliminaci v průběhu různých forem cholestázy, a to především indukované endotoxinem. Současně byla sledována modulace hodnocených procesů vlivem užívaných nebo potenciálních protizánětlivých látek.

Sepse navozená gram-negativními bakteriemi způsobuje akutní ledvinné selhání (AKI) prostřednictvím aktivace imunitní odpovědi, vyvolané lipopolysacharidy (LPS) na jejich povrchu. V této studii jsme se zabývali možností zabránit takovému poškození dvěma účinnými protizánětlivými látkami, dexametasonem a anakinrou, antagonistou na IL-1 receptorech. Biochemické i molekulární znaky renálního poškození byly pozorovány u potkanů, kterým byl aplikován LPS ze *Salmonella typhimurium*, po předchozí premedikaci fyziologickým roztokem, dexametasonem nebo anakinrou. U neléčených endotoxemických potkanů se během 10-ti hodin objevily znaky typické pro renální poškození, chararakterizované sníženou glomerulární filtrací, mikroalbuminurií a sníženou tubulární sekrecí azitromycinu, modelového

substrátu pro transportéry Mdr1 a Mrp2. Premedikace oběma imunosupresivy zmírnila všechny tyto znaky typické pro AKI a došlo k obnovení tubulární sekrece azitromycinu na úroveň kontrolních potkanů. Tento účinek byl spojen s up-regulací bazolaterálních transportérů pro organické anionty, ale ne apikálních Mdr1 a Mrp2, které byly paradoxně po aplikaci obou látek sníženy. Dále, dexametazon zvýšil exkreci žlučových kyselin snížením transportéru pro jejich reabsorpci Asbt. U obou látek došlo ke snížení plazmatických koncentrací cytokinů, podílejících se na vzniku zánětu a snížení koncentrace NO, jako odpověď na redukci exprese iNOS v ledvinách a játrech. Dexametazon i anakinra byly schopny zmírnit příznaky AKI a modulovat změny v expresi transportérů zapojených do renálního vylučování léčiv, které byly navozeny aplikací endotoxinu. V této práci jsme prokázali významnou úlohu IL-1 beta pro rozvoj renálního poškození během sepse.

Dalším krokem bylo objasnění změn žlučové exkrece látek, ke kterým dochází během sepse. Hodnotili jsme protektivní vliv klinicky dostupných chelátorů železa na rozvoj akutního poškození jater po aplikaci endotoxinu, kdy lipopolysacharid byl podáván samostatně nebo po předchozí předléčbě dexrazoxanem (DEX) nebo deferoxaminem (DFO). Přestože obě látky dokázaly snížit obsah železa v játrech, pouze DFO prokázal protektivní účinek proti jaternímu poškození.

K experimentálně často studovaným formám cholestázy patří stav navozený aplikací ethinylestradiolu a porucha provázející nealkoholové ztukovatění jater (NAFLD). U obou forem jsme analyzovali choleretický potenciál boldinu, včetně molekulárních mechanismů. Boldin urychlil tvorbu žluče u potkanů s aplikovaným ethinylestradiolem. Důležitým zjištěním bylo, že po aplikaci boldinu dochází k up-regulaci Bsep se zvýšenou biliární clearance jeho substrátů, žlučových kyselin. V této souvislosti jsme prokázali schopnost boldinu stimulovat FXR, transkripční regulátor Bsep. Tento mechanismus byl potvrzen v následné studii, kde byla cholestáza provázející NAFLD indukovaná aplikací vysokosacharidové diety (HSD) potkanům s dědičnou hypertriglyceridémií (HHTg). Zde boldin zmírnil negativní dopady rozvíjející se NAFLD na biliární exkreci žlučových kyselin i glutathionu. Tato data podporují pozitivní vliv FXR agonistů v terapii NAFLD.

# Abstract

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**Title of doctoral thesis:**

## Influence of inflammation modulation on excretory mechanisms during intrahepatic cholestasis

Intrahepatic cholestasis accompanies several systemic diseases, and can be induced by several drugs. All of its forms are associated with a certain degree of inflammation. The aim of this research was therefore to study changes in hepatic and renal elimination pathways during different forms of cholestasis, especially endotoxin-induced, and to characterize their modulation by administration of currently used or potential anti-inflammatory agents.

One of the most significant alteration of excretory mechanisms develops during sepsis. The status induces acute renal injury through activation of immune response activated by lipopolysaccharides (LPS) on their surface. In this study, we examined the possibilities to prevent such damage by two potent anti-inflammatory drugs, dexamethasone and anakinra, an IL-1 receptor antagonist. Biochemical and molecular signs of renal impairment were observed in rats administered the LPS from *Salmonellatyphimurium*, after pre-treatment with saline, dexamethasone or anakinra. In untreated endotoxemic rats characteristic symptoms of renal damage appeared within 10 hours - such as reduced glomerular filtration, microalbuminuria and reduced tubular secretion of azithromycin, prototype substrate for the transporters Mdr1

and Mrp2. Pre-treatment by both immunosuppressants alleviated all of these hallmarks typical for AKI and returned tubular secretion of azithromycin back to the control level. This effect was associated with up-regulation of basolateral transporters for organic anions. Application of both substances paradoxically reduced apical Mdr1 and Mrp2 transporters. Furthermore, dexamethasone increased renal excretion of bile acid through downregulation of transporter for reabsorption, Asbt. Both agents decreased the plasma concentrations of cytokines involved in the inflammation and decreased the concentration of NO in response to the reduction of iNOS expression in the kidneys and liver. Dexamethasone and anakinra were able to alleviate the symptoms of acute kidney injury and modulate changes in expression of transporters involved in renal excretion of drugs which were imposed due to administration of endotoxin. In this work, we have demonstrated the important role of IL-1 beta in the development of renal impairment during sepsis.

The next step was to elucidate the changes in billiary excretion of drugs during sepsis. We evaluated the protective effect of the clinically available iron chelators in the development of acute liver injury after administration of endotoxin, where lipopolysaccharide was administered, or following pre-treatment with dexrazoxane (DEX) or deferoxamine (DFO). Although both compounds reduced the iron content in the liver, only DFO demonstrated a protective effect against liver damage.

Further, we analyzed the potential choleretic effect of boldin, a natural choleretic agent in healthy and cholestatic rats. Boldin caused up-regulation of Bsep and increased biliary clearance of substrates and bile acids. Furthermore, we described the ability of boldin to stimulate FXR, a transcriptional regulator of Bsep. We confirmed this mechanism in the following study in which cholestasis was induced by high sucrose diet (HSD) applied to rats with hereditary hypertriglyceridemia (HHTg). Boldin alleviated the negative influence of HSD-induced liver steatosis on the biliary excretion of bile acids and glutathione. The data support positive effects of FXR agonists in the therapy of non-alcoholic fatty liver disease.

# Obsah

# 1. Seznam zkratek

| | |
|---|---|
| ABC | ATP-binding cassete |
| AKI | acute kidney injury |
| ALT | alaninaminotransferáza |
| AP-1 | activator protein 1 |
| Asbt | apical sodium dependent bile acid transporter |
| AST | aspartátaminotransferáza |
| ATP | adenosine triphosphate |
| BCRP | breast cancer resistance protein |
| BSEP | bile salt export pump |
| CBP | CREB binding protein |
| CD14 | cluster of differentiation 14 |
| COX | cyclooxygenase |
| CREB | cAMP responsive element binding protein |
| CYP450 | cytochrome P450 |
| DEX | dexrazoxan |
| DFO | deferoxamin |
| EDTA | ethylenediaminetetraacetic acid |
| FXR | farnesoid x receptor |
| GFR | glomerular filtration rate |
| GK-GR | komplex glucocorticoid – glucocorticod receptor |
| GR | glucocorticoid receptor |

| | |
|---|---|
| GRE | glucocorticoid response element |
| GSH/GSSG | redukovaný glutation/oxidovaný glutation |
| HHTg | hereditary hypertriglyceridemic |
| HSD | vysokosacharidová dieta |
| ICAM-1 | intracellular adhesion molecul 1 |
| IL | interleukin |
| IL-1R | interleukin 1 receptor |
| IL-1Ra | interleukin 1 receptor antagonist |
| INF gamma | interferon gamma |
| iNOS | inducible nitric oxide synthase |
| LPS | lipopolysaccharide |
| MATE | multidrug and toxin extrusion protein |
| MCP-1 | monocyte chemoattractant protein-1 |
| MDR | multidrug resistance protein, P-gp |
| MIC | minimální inhibiční kncentrace |
| MIF | macrophage migration inhibitory factor |
| MRP | multidrug resistance-associated protein |
| NAFLD | non-alcoholic fatty liver disease |
| NF-kappa B | nuclear faktor kappa B |
| NK | natural killer |
| NO | nitric oxide |
| Ntcp | $Na^+$-taurocholate cotransporting polypeptide |
| OATP | organic anion-transporting polypeptide |

| | |
|---|---|
| OATs | organic anion transporters |
| OCTN | organic cation/carnitine transporter |
| OCTs | organic cation transporters |
| PAMPs | pathogen-associated molecular patterns |
| P-gp | P-glycoprotein |
| SIRS | systemic inflammatory response syndrome |
| SLC | solute carrier |
| TGF beta | transforming growth factor beta |
| TLRs | Toll-like receptors |
| TNF | tumor necrosis factor |
| TNFRs | tumor necrosis factor recepors |
| VCAM-1 | vascular cell adhesion protein 1 |

# 2. Úvod

Sepse je závažný stav podmíněný excesivní systémovou odpovědí imunitního systému (SIRS, systemic inflammatory response syndrome) na přítomnost infekce. Pokud nedojde k adekvátnímu léčení, situace se může rozvinout do těžké sepse spojené s hypotenzí, hypoperfúzí tkání a orgánovou dysfunkcí. Hypotenze postupně přestane reagovat na adekvátní tekutinovou resuscitaci a rozvíjí se septický šok s 60 % mortalitou.

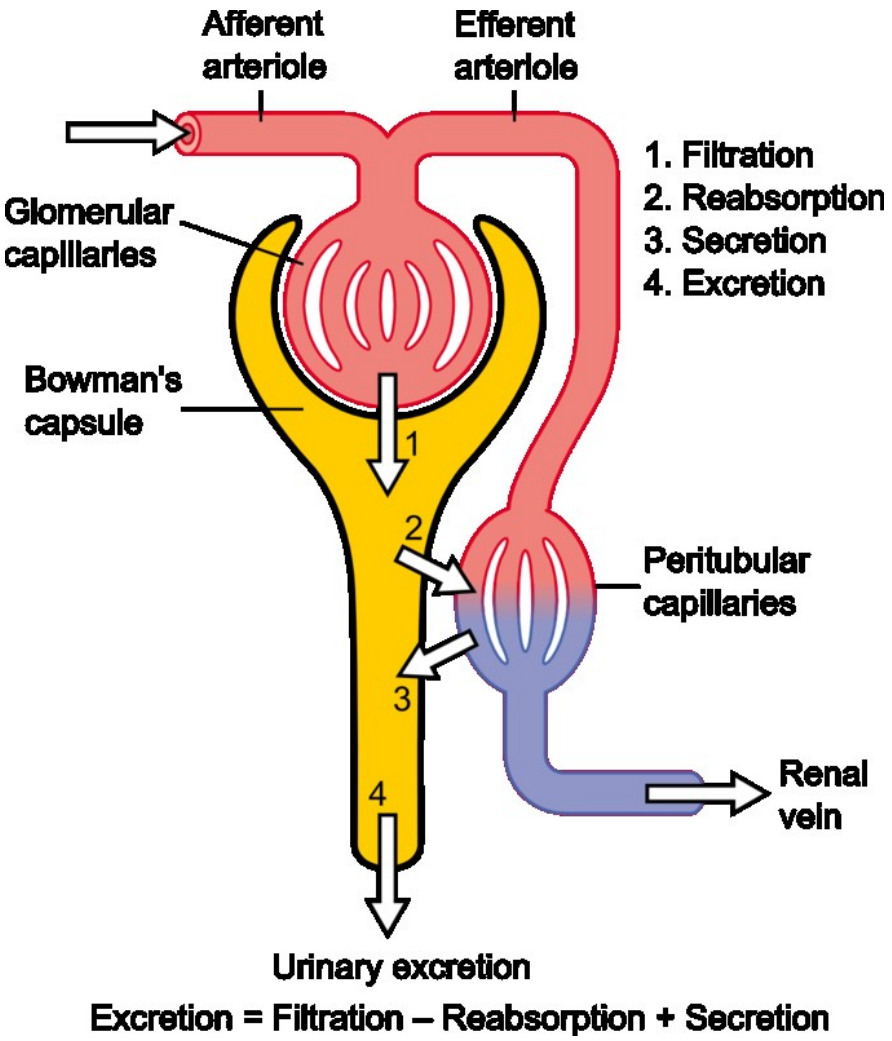
Jednou z nejčastějších komplikací, která dále zhoršuje prognózu nemocných v sepsi, je akutní poškození ledvin (AKI), ke kterému dochází jak v důsledku hypoperfuze orgánu, tak i přímou interakcí struktur invadujícího mikroorganismu s buňkami glomerulů a tubulů. Kromě výrazného poklesu glomerulární filtrace tak dochází i ke změnám exprese a funkce důležitých transportérů, které se podílejí na eliminaci řady endo- i xenobiotik včetně léčiv a jejich metabolitů. Důsledkem narušení těchto základních eliminačních dějů je možnost nežádoucí kumulace léčiv, která může podpořit jejich toxicitu. Z toho důvodu je nezbytně nutné podrobně popsat změny v renálních eliminačních cestách během sepse a charakter jejich modulace stávajícími nebo potenciálními terapeutiky.

Molekulární podstata iniciace SIRS vychází z interakce mikrobiálních antigenů s receptory na povrchu buněk nespecifické imunitní odpovědi, ale i exekutivních buněk jednotlivých tkání (např. hepatocytů a buněk renálních tubulů). Především se jedná o aktivaci Toll-like receptorů cirkulujícími endotoxiny (LPS, lipopolysacharid) baktérií s následnou stimulací jaderného transkripčního faktoru NF kappa B, která vede ke zvýšené produkci prozánětlivých cytokinů (TNF alfa, IL-1 beta) a oxidu dusnatého. Nedílnou součástí léčby sepse se proto zejména v závažnějších stadiích staly glukokortikoidy, které významně tlumí zánět právě potlačením NF kappa B kaskády. Příznivě tak modulují patologické změny v jednotlivých orgánech. Nadějnou možností se rovněž jeví použití více selektivní blokády jednotlivých cytokinů, zejména IL-1. Syntetický antagonista receptoru pro IL-1 byl mimo jiné účinný v prevenci deregulace důležitých jaterních transportérů pro léčiva navozená endotoxiny. Nicméně vliv těchto protizánětlivých látek na ledvinnou eliminaci léčiv prozatím prozkoumán nebyl. Proto se předmětem této práce stalo hodnocení efektu dexametazonu a anakinry (antagonista IL-1 receptoru) na průběh eliminace léčiv jak v ledvinách, tak okrajově i v játrech.

# 3. Teoretická část

## 3.1. Renální eliminace léčiv

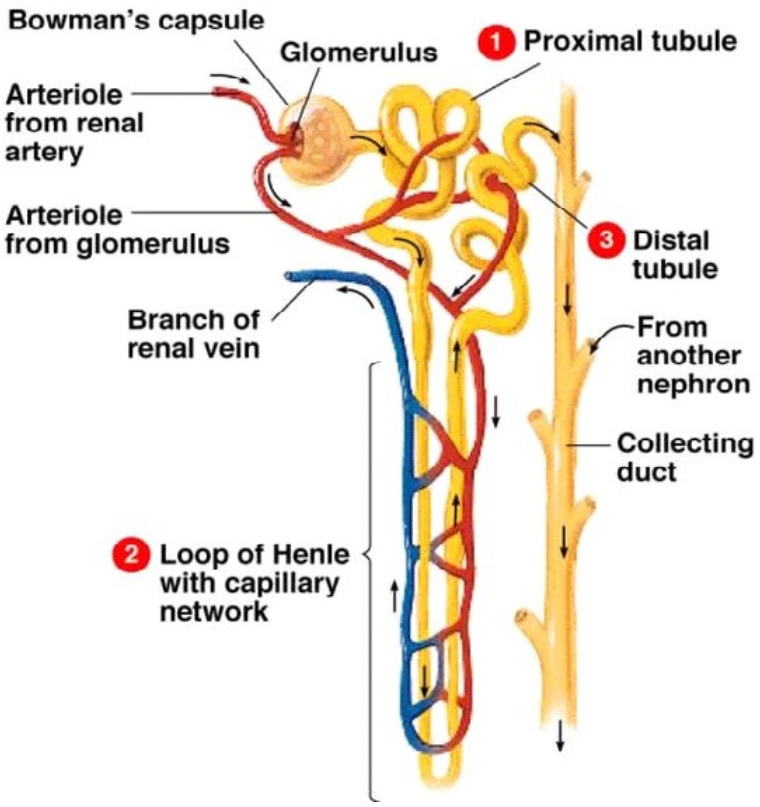
Lidské tělo je neustále vystavováno potenciálně škodlivým látkám, včetně léčiv, toxinů a endogenních metabolitů (1). Pro zajištění vnitřního prostředí je proto nutná přítomnost mechanismů zajišťujících eliminaci takových látek z organismu. Tyto procesy představují kombinaci metabolických a exkrečních drah, do kterých látky vstupují v závislosti na své struktuře a z ní vyplývajících fyzikálně-chemických vlastností. Terminální fází eliminace je pak exkrece, kdy dochází k vyloučení látek z organismu. Orgány, které se zapojují do tohoto děje, jsou zejména ledviny, plíce, játra a střeva (2). Z hlediska podílu na eliminaci jednotlivých látek a jejich metabolitů mají primární roli ledviny. To je podmíněno existencí třech významných procesů, které zprostředkují exkreci látek do moče: glomerulární filtrace, tubulární sekrece a tubulární reabsorpce – Obr. 1. (3). Podíl každého z těchto procesů na celkové renální eliminaci závisí na chemických vlastnostech dané látky. Základní podmínkou pro vyloučení do moče je rozpustnost ve vodě. Další faktory ovlivňující renální exkreci zahrnují zejména vazebnost na plazmatické bílkoviny, distribuční objem léčiva, pH moče, rychlost glomerulární filtrace a přítomnost transportérů pro aktivní tubulární sekreci a reabsorpci (4).




Obr. 1. Renální exkrece látek: filtrace malých volných molekul skrz póry glomerulárních kapilár, reabsorpce liposolubilních a neioizovaných látek a aktivní sekrece látek v tubulární části nefronu. Upraveno dle Boundless (5).

Ledvina (ren, nefros) je párový orgán, oválného (fazolovitého) tvaru, uložená po obou stranách bederní páteře v retroperitoneálním prostoru v blízkosti dvou hlavních cév, aorty a dolní duté žíly. Je to nejlépe perfundovaný orgán v těle a vysoká rychlost průtoku krve je pro jeho správnou funkci nezbytná. Krev protéká ledvinami rychlostí 1,2 l/min. Základní stavební a funkční jednotkou ledviny je nefron, který se skládá z glomerulu a z ledvinových kanálků – Obr. 2. Glomerulus tvoří konvolut kapilár a je obklopen dvouvrstvým Bowmanovým pouzdrem. Mezi parietálním listem tvořícím vnější obal glomerulu a vnitřním listem viscerálním přiléhajícím na kličky jednotlivých kapilár je kapsulární prostor, který pokračuje

do tubulu a tudy odtéká primární přefiltrovaná moč. Ledvinové tělísko má dva póly – cévní pól, kde vstupuje arteriola afferens a vystupuje arteriola efferens a močový pól, kudy odtéká primární moč do proximálního tubulu, Henleovy kličky, distálního tubulu a sběrného kanálku (6).

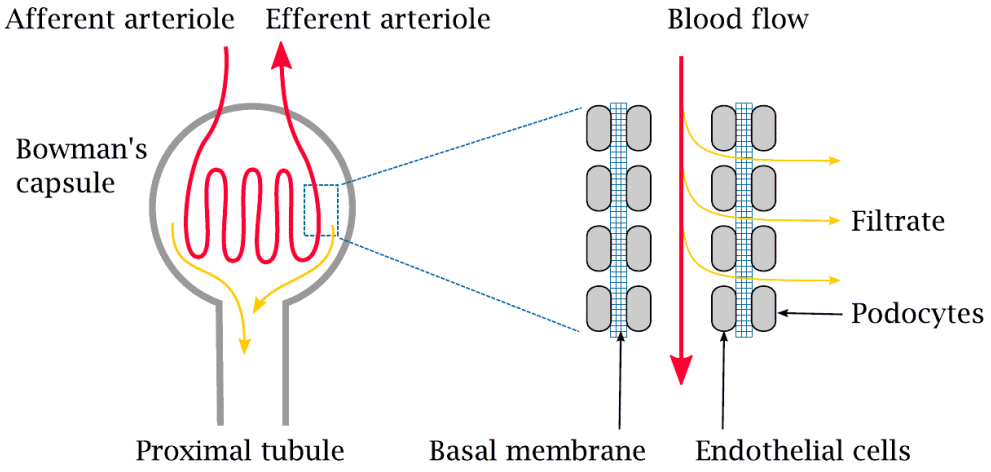



Obr. 2. Stavba nefronu. Glomerulus s Bowmanovým pouzdrem, proximální tubulus, Henleova klička, distální tubulus a sběrný kanálek. Upraveno dle Gallery nephron diagram (7).

### 3.1.1. Glomerulární filtrace

První fází tvorby moče je filtrace krevní plazmy přes glomerulární kapiláry do Bowmanova pouzdra, kde se vytváří primární moč (glomerulární filtrát). Membrána glomerulárních kapilár je tvořena ze tří hlavních vrstev: endotelem fenestrovaných kapilár, bazální membránou, která je vně obklopena vrstvou epiteliálních buněk (podocytů) – Obr. 3.

Stejně jako u kapilár krevního oběhu i zde tvoří bazální membrána skutečné molekulární síto filtrace a všechny tři jmenované vrstvy tvoří filtrační bariéru (4). Stěna glomerulární kapiláry je sestavena tak, aby umožňovala vysoký stupeň filtrace krevní plazmy, při současném omezení průchodu látek, které mají poměrné velkou molekulární hmotnost (2). Selekce je ve filtraci velmi důležitá, neboť zabraňuje filtraci plazmatických bílkovin (zejména albuminu), které jsou nezbytné pro udržení koloidně osmotického (onkotického) tlaku v cévách, tedy i objemu plazmy.




Obr. 3. Struktura glomerulárních kapilár. Upraveno dle Palmer et al. (4).

Glomerulární filtrace (GF) je jednosměrná difúze, která zajišťuje průchod volného léčiva (látky) z krve do lumen ledvinných tubulů a je ovlivněna řadou faktorů, zejména pak renálním krevním průtokem, molekulární velikostí látek, distribučním objemem látek, vazebností na plazmatické bílkoviny, nábojem a tvarem a kvalitou bariéry, která může být změněná během různých onemocnění. Nabité molekuly o stejné molekulové hmotnosti, jako látky neutrální, se obecně budou filtrovat pomaleji. Týká se to především negativně nabitých molekul, jejichž filtrace je značně omezená. Hlavním důvodem je pravděpodobně interakce mezi filtrovanou molekulou a negativním nábojem stěny glomerulární kapiláry. Všechny výše zmiňované faktory, které ovlivňují glomerulární filtraci (GFR), mohou také změnit clearance filtrovaných látek. Například zánětlivé onemocnění glomerulárních kapilár může zvýšit GFR a tím i filtraci léčiv. Většina léčiv je navíc alespoň částečně vázaná na plazmatické bílkoviny, tj.

jejich skutečná filtrace bude menší než filtrace teoretická, určená z celkové koncentrace v plazmě (2,4).

Farmakokinetickým parametrem popisujícím eliminaci léčiv v ledvinách je renální clearance ($CL_R$) (5,6). Definujeme ji jako objem plazmy, který je za jednotku času očištěn od sledované látky – vzorec výpočtu viz níže. Pro látky, které jsou pouze filtrovány, nejsou významně vázány na plazmatické bílkoviny a nejsou secernovány ani zpětně vstřebávány v tubulech, je renální clearance totožná s GFR. Prototypem takové látky je inulin, kde platí $CL_{Rinulin} = GFR$.

$$CL_R = R_R/C_p$$

$CL_R$ ….. renální clearance látky (ml/min)

$R_R$ ….. rychlost renální exkrece (mg/min)

$C_p$ ….. koncentrace látky v plazmě (mg/ml)

Hodnota GF u zdravých jedinců dosahuje přibližně 125 ml/min a může být výrazně snížena u osob trpících chronickou renální insuficiencí nebo akutním renálním selháváním. Zhruba 99 % primární moče je reabsorbováno zpět do extracelulární tekutiny na podkladě aktivních a pasívních dějů probíhajících v tubulech (8).

### 3.1.2. Renální reabsorpce

Renální reabsorpce může zahrnovat děje jak pasivní (pasivní zpětná difúze), tak aktivní (aktivní transport látek přes membránu). U většiny organických aniontů a kationtů probíhá zpětné vstřebávání jako pasivní zpětná difúze. Míra této reabsorpce je přímo úměrná koncentračnímu gradientu dané látky a její rozpustnosti v tucích a nepřímo úměrná stupni ionizace v závislosti na pKa sledované látky a pH moči. pH moči (4,5-8) může proto výrazně ovlivnit rychlost pasivního zpětného vstřebávání. Modulaci pH moče lze v této souvislosti využít pro podporu exkrece během intoxikací. Např. alkalizace moči může být využita ke snížení pasivní reabsorpce kyselých léčiv typu barbiturátů. Koncentrace látky, a tím i koncentrační gradient mezi močí a intersticiem ledviny, se při postupu v tubulárním systému

nefronu postupně zvyšuje v důsledku reabsorpce vody a iontů (2). K pasivní zpětné difúzi proto dochází především v distálním tubulu a sběrném kanálku, kde je koncentrační gradient nejvyšší (9).

Aktivní reabsorpce je zprostředkovaná činností transportních proteinů – přenašečů, na apikální a bazolaterální straně buněk výstelky, především proximálních tubulů. Transportní proteiny vykazují substrátovou specifitu a kapacitu, která může být omezena saturací nebo inhibicí. Pro činnost přenašečů je nutná buď dodávka energie ve formě ATP, nebo je hnací sílou současný transport jiné látky, která přechází po směru koncentračního gradientu (např. $Na^+$). Zejména v prvním případě je pak možný přenos látky proti koncentračnímu gradientu. Další možností je facilitovaná difuze, kdy je léčivo transportováno bez nutnosti přísunu energie po směru koncentračního gradientu. Tyto děje jsou důležité pro reabsorpci živin, jako jsou aminokyseliny, nukleosidy a glukóza – Obr. 4. (10). U některých látek navíc dochází k obousměrnému aktivnímu transportu přes apikální membránu proximálního tubulu. Tento mechanismus byl popsán pro některé organické anionty (např. kyselinu močovou), kdy je látka aktivně reabsorbovaná i secernovaná (2).

### 3.1.3. Renální sekrece

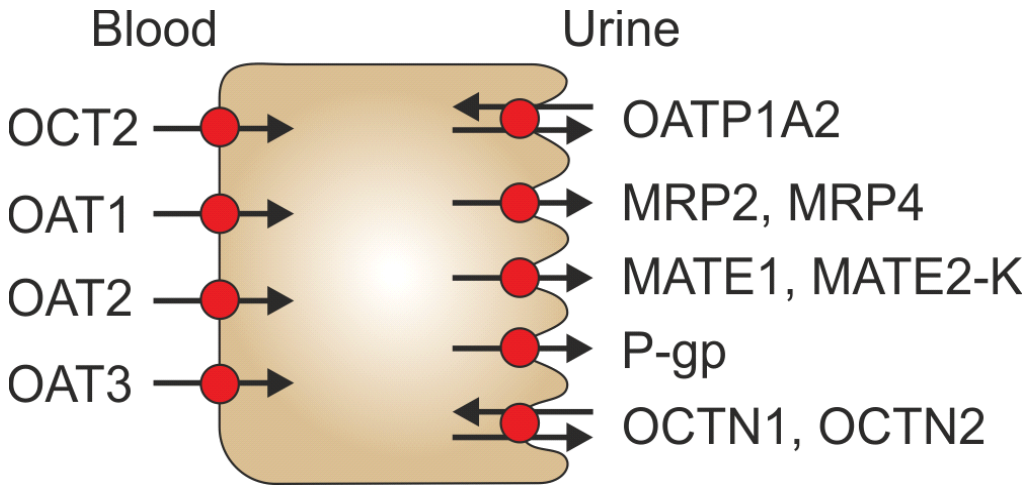
Substrátů pro aktivní transportní sekreční systém v proximálním tubulu existuje celá řada. Tento transportní systém, který aktivně přenáší látky z krve do lumen tubulů, zahrnuje sekreci organických aniontů a organických kationtů. Tyto dva transportní systémy jsou na sobě nezávislé (2).

## 3.2. Transportéry podílející se na renální eliminaci léčiv

V posledních 20- ti letech byl identifikován velký počet membránových transportních proteinů, které mají nezastupitelnou roli v absorpci, distribuci a eliminaci látek (léčiv). Transportéry lze rozdělit do dvou hlavních tříd, SLC (solute carrier) a ABC (ATP-binding casette). SLC i ABC transportéry jsou hojně zastoupeny v celém organismu a podílejí se na transportu široké škály látek. V závislosti na směru, ve kterém transportéry (specifické proteiny)

přenášejí substrát přes buněčnou membránu, je rozdělujeme na influxní (vstup látek do buněk) a efluxní (výdej látek z buněk) (11).

ABC transportéry se řadí mezi efluxní. Zprostředkovávají aktivní transport látek z intracelulárního do extracelulárního prostředí za spotřebování energie získané hydrolýzou ATP. Naopak SLC transportéry patří převážně mezi influxní, kdy usnadňují vychytávání nebo přenos substrátu do buňky. A to buď usnadněnou difúzí, nebo aktivním transportem. Některé SLC transportéry jsou obousměrné, v závislosti na koncentračním gradientu substrátu. Je důležité si uvědomit, že souhra mezi transportéry na apikální (luminální) a bazolaterální (krevní) membráně polarizovaných buněk (ledvinné tubulární buňky, hepatocyty, enterocyty atd.) je rozhodující pro určení směru pohybu léčiva v orgánech, jako jsou játra, ledviny, střeva. Všechny tyto transportéry ovlivňují farmakokinetický profil svých substrátů v organismu a dále se podílejí na vylučování látek z oběhu do žluči, moči nebo lumen střeva. Přehled základních substrátů pro jednotlivé transportéry nabízí Tab. 1. V dalším textu bude pozornost věnována zejména transportérům důležitým pro eliminaci léčiv v ledvinách, které v přehledu demonstruje Obr. 4. (11).




Obr. 4. Přehled renálních transportérů. Upraveno dle Kathleen M. Giacomini et al. (12).

### 3.2.1. SLC transportéry

Pro léčiva jsou z této skupiny důležité především transportéry z rodin SLC22 a SLCO – Tabulka 1. SLC22 sdružují transportéry pro organické anionty (OAT), organické kationty (OCT) a pro organické zwitteriony/kationty (OCTN). Charakteristická je pro ně široká substrátová specifita. Hlavní zástupci této rodiny transportérů se nalézají hlavně v játrech, ledvinách a střevech. Další rodinou transportérů jsou SLCO, kam patří polypeptidové transportéry pro organické anionty (OATPs). V poslední době byla navíc v kinetice léčiv popsaná důležitost transportérů multidrug and toxin extrusion (MATE) ze skupiny SLC47 (11).

*3.2.1.1. OATs*

Transportéry pro organické anionty (OATs) hrají velmi důležitou roli v ledvinné eliminaci léčiv, jelikož negativně nabitá glomerulární membrána omezuje filtraci aniontů. Tyto transportéry jsou proto lokalizovány především v ledvinách, ale některé najdeme i v játrech, placentě a mozku. V ledvinách umožňují především influx organických aniontů z krve do buněk proximálních tubulů. Pracují na základě antiportu, kdy příjem organického aniontu je spojen s výměnou za jiné organické anionty přenášené ven z buňky. K tomuto ději dochází bez spotřeby energie - hybnou silou je intracelulární-extracelulární koncentrační gradient. Vychytávání látek na bazolaterální membráně proximálního tubulu je zprostředkováno a regulováno převážně OAT1 a OAT3. Oba transportéry se v substrátové specifitě překrývají, a i jejich mechanismus transferu je stejný. Organický aniont je do buňky přijímán výměnou s intracelulárním alfa-ketoglutarátem (13). OAT3 v porovnání s OAT1 má větší substrátovou specifičnost, je více exprimován v ledvinách a hraje výraznější roli v renálním transportu organických aniontů u lidí (10,14).

*3.2.1.2. OCTs, OCTNs*

Další skupinou SLC22 jsou transportéry pro organické kationty, OCTs a OCTNs. Specializují se na přenos organických kationtů přes bazolaterální membránu do buňky facilitovanou difúzí, po směru elektrochemického potenciálu. Oproti tomu transport přes kartáčový lem probíhá jako antiport organický kation – proton. Tyto dva mechanismy jsou důležité pro sekreci organických kationtů z krve do moče. Rozlišujeme 2 skupiny transportérů pro kationty: OCTs (OCT1-OCT3) a OCTNs (OCTN1, OCTN2). Všechny jsou ve větším či menším množství zastoupeny v ledvinách a hrají důležitou roli v eliminaci mnoha léčiv (15). Např. OCT1 je u lidí nejvíce exprimován v játrech a v menším rozsahu pak ve střevech a úplně

nejmenší zastoupení je v ledvinách. Naopak OCT2 je nejvíce zastoupen v ledvinách, méně pak ve střevě a v játrech úplně chybí (16).

*3.2.1.3. OATPs*

Další rodinou transportérů jsou SLCO, kam patří polypeptidové transportéry pro organické anionty (OATPs). Podílejí se na vychytávání substrátů o větší molekulové hmotnosti (MW je větší než 450 Da) a relativně lipofilních organických aniontů. Jejich substrátová specifita je široká, zahrnuje amfipatické organické sloučeniny, jako soli žlučových kyselin, hormony štítné žlázy a steroidní konjugáty (17). Jejich přesný mechanismus činnosti je nejasný. Má se za to, že pracují jako obousměrné antiporty závislé na pH, které jsou stimulovány kyselostí extracelulárního prostředí (18–21). Některé studie naznačují, že fungují jako elektroneutrální výměníky. OATPs mohou vyměňovat své substráty za intracelulární bikarbonát (22,23), glutathion (24,25) nebo konjugáty glutathionu (20). Jejich rozložení v tkáních je různé. Některé izoformy jsou zastoupeny skoro ve všech tkáních a u některých je exprese omezena pouze na jeden orgán. Bylo popsáno 11 lidských OATPs, které jsou zařazeny do 6 rodin podle identity sekvencí aminokyselin, které obsahují. Mezi nejvíce prozkoumané a popsané patří rodina 1, která obsahuje OATP1A2, OATP1B1, OATP1B3 a OATP1C1 (26). OATP1A2 hraje hlavní roli v absorpci, distribuci a exkreci xenobiotik. V játrech je výhradně exprimován v cholangiocytech (27), a může se podílet na reabsorpci xenobiotik, které jsou exkretovány do žluče. V ledvinách je OATP1A2 exprimován na apikální straně membrány v distální části nefronu (27), kde je zodpovědný za reabsorpci léčiv z moči nebo naopak za jejich sekreci. OATP1B1, OATP1B3 se nachází primárně na bazolaterální straně hepatocytů (28–30). OATP1B1 je exprimován po celém lalůčku, zatímco OATP1B3 se nachází hlavně okolo centrální žíly (31). Význam OATP1B/1B3 v ledvinách je zatím nejasný.

*3.2.1.4. MATE1, MATE2*

MATE1 (multidrug and toxin extrusion protein) se nachází v kartáčovém lemu na apikální membráně buněk proximálních tubulů a na apikální membráně v hepatocytech. Podílejí se na transportu látek přes membránu jako antiport organický kation/$H^+$. Hraje důležitou roli v renální a biliární exkreci exogenních a endogenních kationtů, včetně léčiv (32,33). MATE1 v játrech tvoří funkční celek s bazolaterálním OCT1 transportérem, se kterým zprostředkovává biliární exkreci mnoha kationtových léčiv a jejich metabolitů (32). V proximálním tubulu ledvin spolupracuje MATE1 s bazolaterálním OCT2 transportérem a

účastní se společně na sekreci řady organických kationtů (15,32). Kromě jater a ledvin je MATE1 exprimován mimo jiné v srdci, nadledvinách, varleti a kosterním svalstvu. V jiných orgánech, než jsou ledviny a játra, je fyziologická role MATE1 méně známá (34). MATE2 je exprimován v kartáčovém lemu proximálních tubulů a bylo zjištěno, že se nachází pouze v ledvinách. Tento renální transportér je zodpovědný za přenos léčiv přes kartáčový lem. Hraje důležitou roli v tubulární sekreci léčiv (34). Podílí se na renální eliminaci léčiv ve spolupráci s OAT2.

| Gen | protein | mechanismus | hlavní tkáňová distribuce | membránová lokalizace | příklady substrátů |
|---|---|---|---|---|---|
| ***SLC22* rodina** | | | | | |
| *SLC22A1* | OCT1 | OC uniport | Střevo | BLM | acyklovir, metformin, |
| | | | Játra | AM | cimetidin, zidovudin |
| *SLC22A2* | OCT2 | OC uniport | Ledviny | BLM | cimetidin, famotidin, ranitidin, metformin |
| *SLC22A6* | OAT1 | DK/OA antiport | Ledviny | AM | adefovir, acyklovir, metotrexát, ketoprofen, ibuprofen, PAH, ranitidin |
| *SLC22A7* | OAT2 | OA antiport | Játra | BM | erytromycin, allopurinol, PAH, teofylin, salicyláty, |

| | | | | | |
|---|---|---|---|---|---|
| | | | | | ranitidin |
| *SLC22A8* | OAT3 | DK/OA antiport | Ledviny | LM | tetracyklin, pravastatin, olmesartan, PAH, furosemid, metotrexát |
| ***SLC47* rodina** | | | | | |
| *SLC 47A1* | MATE1 | $H^+$/OC antiport | Játra<br>Ledviny | KM<br>LM | metformin, cimetidin, prokainamid |
| *SLC47A2* | MATE2 | $H^+$/OC antiport | Ledviny | LM | metformin, cimetidin, prokainamid |
| ***SLCO* rodina** | | | | | |
| *SLCO1A2* | OATP1A2 | OA antiport | Střevo<br>Ledviny | LM<br>LM | indometacin, rosuvastatin, enalapril, levofloxacin, metotrexát |

Tabulka 1. Přehled SLC transportérů důležitých pro renální eliminaci léčiv.

Zkratky: OC – organické kationty, OA – organické anionty, BLM –bazolaterální membrána, AM – apikální membrána, LM – laminární membrána (kartáčový lem), KM – kanalikulární membrána, DK – dikarboxylát, PAH – p-aminohippurová kyselina, upraveno dle Russel et al. (10).

### 3.2.2. ABC transportéry

ABC transportéry – Tabulka 2. hrají důležitou roli při absorpci, distribuci a eliminaci léčiv, jelikož se hojně vyskytují zejména na apikálních membránách buněk s bariérovou nebo exkreční funkcí. Některé z nich jsou rovněž spojovány s fenoménem mnohočetné lékové rezistence (např. P-gp/MDR1 – multidrug resistence protein) v důsledku přítomnosti v membráně nádorových buněk. Mají malou molekulovou hmotnost 150-200 kDa a zajišťují export látek (léčiv) ven z buňky za spotřeby energie, kterou získávají hydrolýzou ATP na ADP, tj. patří mezi transportéry s efluxní ATPázovou aktivitou (35).

#### *3.2.2.1. P-gp/MDR1*

P-glykoprotein (P-gp) patří mezi hlavní transportéry rodiny ABC. Jeho substrátová specifita je neobvykle velmi široká. Dokáže rozpoznat stovky sloučenin od malých molekul (350 Da) až po polypeptidy (4000 Da). P-gp je exprimován v mnoha tkáních, jako střeva, játra, ledviny, plíce, placenta. P-gp je jeden z hlavních efluxních transportérů, zajišťující transport látek z intracelulárního do extracelulárního prostředí přes apikální/luminální membránu buněk. Zapojuje se do ledvinné a biliární exkrece, kdy chrání organismus před potenciálně škodlivými látkami včetně řady léčiv. V enterocytech naopak působí proti vstřebávání substrátů, a tím modifikuje jejich biologickou dostupnost (36).

#### *3.2.2.2. BSEP*

Podobný P-gp je bile salt export pump (BSEP) exprimovaný na apikální membráně jater (v malém množství zastoupen i v ledvinách), který je zodpovědný za vylučování (export) monovalentních žlučových kyselin do žluči (37). BSEP se zdá být klíčovým transportérem u léčivy navozené cholestázy. Látky jako glibenklamid nebo rifampicin mohou inhibovat BSEP, což vede k intracelulární akumulaci solí žlučových kyselin, ke snížení toku žluče a v konečném důsledku k poškození jater (37).

#### *3.2.2.3. MRP*

Dále mezi ABC rodinu transportérů řadíme skupinu multidrug resistence-associated proteinů (MRP). MRP transportéry zprostředkovávají transport organických aniontových sloučenin včetně glutathionu, sulfátových konjugátů, glukuronidů a dokonce i některé kationtové substráty za přítomnosti redukovaného glutathionu (38).

Najdeme je především ve střevě, játrech a ledvinách, v menším zastoupení i v ostatních tkáních (placenta, mozek) (39,40). V ledvinách hraje nejvýznamnější roli MRP2 a MRP4 v sekreci na apikální membráně buněk proximálních tubulů. Menší roli má MRP3, který je lokalizován na bazolaterální membráně a zprostředkuje eflux do intersticiální tekutiny.

*3.2.2.4. BCRP*

BCRP (Breast cancer resistence protein) patří mezi důležité efluxní transportéry. Podílí se na vylučování xenobiotik a látek toxických pro organismus ve střevě, hepatocytech i tubulárních buňkách a brání v penetraci látek přes placentální a hematoencefalickou bariéru (41). V současné době je považován za jeden ze tří hlavních transportérů způsobujících lékovou rezistenci u savců. S tím souvisí i jeho tkáňová distribuce, která odráží jeho důležitou roli v ochraně buněk proti potencionálně cizorodým toxickým látkám. Mezi přirozeně vyskytující se xenobiotika, u kterých hraje BCRP důležitou protektivní roli, patří i mutageny a karcinogeny. Funguje i jako efluxní pumpa pro velké množství léčiv, včetně chemoterapeutik (42).

Některé mechanismy zapojené do renální eliminace léčiv citlivě reagující na různé patofyziologické situace. To se pak může projevit změnami kinetiky a potažmo dynamiky podávaných léčiv. Proto je nutné tyto procesy studovat, aby bylo možné dané změny predikovat a terapii dle toho individualizovat. Jedním z velmi častých závažných zdrojů významné poruchy renálních eliminačních mechanismů je sepse. Další text se proto bude věnovat dané problematice.

| Gen | protein | mechanismus | hlavní tkáňová distribuce | membránová lokalizace | příklady substrátů |
|---|---|---|---|---|---|
| ***ABCB* rodina** | | | | | |
| *ABCB1* | MDR1 | efluxní transportér | střevo | LM | daunorubicin, levofloxacin |
| | | | játra | KM | digitoxin, sumaptriptan |
| | | | ledviny | LM | karbamazepin, amiodaron |
| ***ABCC* rodina** | | | | | |
| *ABCC2* | MRP2 | efluxní transportéry | střevo | LM | doxorubicin, cisplatina |
| | | | játra | KM | PAH, konj. glutathion |
| | | | ledviny | LM | indinavir, metotrexát |
| *ABCC3* | MRP3 | efluxní transportéry | střevo | BLM | metotrexát, |
| | | | játra | AM | glukuronidové konjugáty |
| | | | ledviny | BLM | |
| *ABCC4* | MRP4 | efluxní transportéry | střevo | LM | adefovir, tenofovir, |
| | | | játra | AM | metotrexát, furosemid |
| | | | ledviny | LM | cefazolin, PAH |

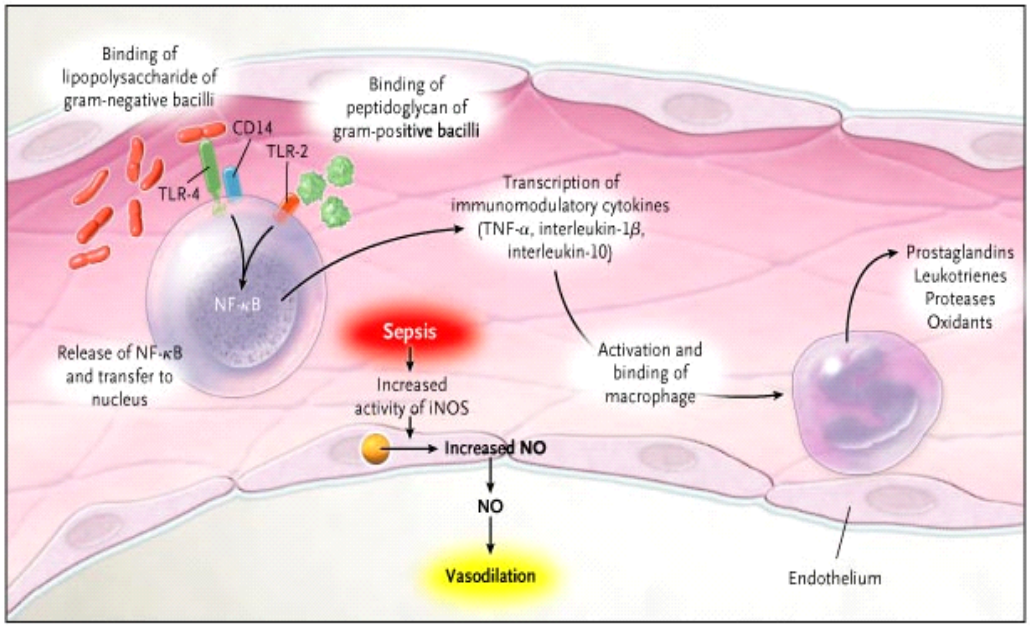
Tabulka 2. Přehled ABC transportérů důležitých v renální eliminaci léčiv.

Zkratky: BLM –bazolaterální membrána, AM – apikální membrána, LM – laminární membrána (kartáčový lem), KM – kanalikulární membrána, PAH – p-aminohippurová kyselina, upraveno dle Russel et al. (10).

## 3.3. Sepse jako zdroj variability renální eliminace léčiv

### 3.3.1. Patofyziologie sepse

Sepsí se označuje život ohrožující stav způsobený především imunitní odpovědí organismu na přítomnost infekčního agens. Rozvíjí se tzv. syndrom systémové zánětlivé odpovědi (SIRS, systemic inflammatory response syndrome), což je stav charakterizovaný dvěma či více z následujících kritérií: a) tělesná teplota > 38 °C nebo < 36 °C; b) tepová frekvence > 90/min; c) dechová frekvence > 20/min nebo $pCO_2$ < 32 mmHg; d) počet leukocytů > 12 tis./µl nebo < 4 tis./µl nebo počet nezralých forem přesahuje 10% (43). Primární roli v rozvoji sepse hrají mechanismy přirozené imunity, které jsou také rozhodující pro aktivaci a modulaci pozdní antigen-specifické imunitní odpovědi. Proces je iniciován antigeny infekční částice (nejčastěji bakteriální), které stimulují Toll-like receptory (TLRs) na povrchu buněk imunitního systému (44), ale i buněk jednotlivých tkáních. TLRs jsou transmembránové proteiny se schopností aktivovat signální dráhy, spouštět vyplavování cytokinů, aktivovat neutrofily a stimulovat endoteliální buňky. TLRs jsou základními receptory patřícími do skupiny PAMPs (pathogen-associated molecular patterns) receptorů v systému přirozené imunity. U savců bylo rozpoznáno 11 různých TLRs. Nejvíce studovaným je TLR4, který reaguje na nejsilnější stimulant přirozené imunity, gram-negativní bakteriální endotoxin, což jsou lipopolysacharidové (LPS) fragmenty jejich zevní membrány (45). Navázání LPS na TLRs vede k aktivaci buněk a sekreci prozánětlivých mediátorů. Klíčovým krokem intracelulárního přenosu signálu je aktivace transkripčních faktorů, které se translokují do jádra a modulují transkripci cílových genů. Hlavním transkripčním faktorem je zde NF-kappa B, který up-reguluje transkripci genů pro mediátory zánětu, jako je TNF-alfa, interleukiny, COX2 a iNOS. Tyto mediátory působí na cévní endotel a hladkou svalovinu, což způsobuje vazodilataci, zvýšenou cévní permeabilitu a přesun neutrofilů do instersticia tkání - Obr 5. Suma všech těchto patofyziologických dějů pak podmiňuje klinickou závažnost sepse. V terapii sepse se proto kromě symptomatologické terapie hypotenze zvažuje i zásah do LPS-TLR-cytokinové kaskády s cílem modulovat imunitní odpověď (46).




Obr. 5. Sepse navozená endotoxiny uvolněných z buněčné stěny G$^-$ mikroorganismů. Endotoxin je lipopolysacharid, který se váže na TLR na povrchu makrofágů, dochází k aktivaci NF-kappa B. Následuje uvolňování prozánětlivých mediátorů zánětu provázené změnou morfologie a funkce různých orgánů. Upraveno podle Angus et al. (47).

### 3.3.2. Cytokiny

Cytokiny jsou malé molekuly bílkovinné povahy (molekulární hmotnost do 40 kDa), které jsou produkovány, aby lokálně modulovaly vznik a rozvoj přirozené i specifické imunitní odpovědi. Primárně dochází k uvolnění TNF-alfa a IL-1beta, což je záhy následováno produkcí prozánetlivých (IL-1, IL-6, IL-12, TNF-alfa, IFN-gamma a MIF) i protizánětlivých mediátorů, jakými jsou IL-10, IL-1 RA (receptor antagonist), TGF-beta a IL-4, které se starají o nastavení imunitní rovnováhy (48). Tabulka 3. prezentuje význam jednotlivých cytokinů v regulaci zánětlivé odpovědi během sepse.

| Cytokiny | hlavní funkce | změna/zapojení do onemocnění | terapeutické využití |
|---|---|---|---|
| **TNF-alfa** | aktivace buněk imunitního systému | ateroskleróza, RA | sTNFRs |
| | navozuje horečku a koagulaci | Alzheimerova choroba, autoimunitní onemocnění | TNFR inhibitory |
| | způsobuje kachexii | nádorová onemocnění | anti-TNF Ab |
| **IL-1** | navozuje horečku a koagulaci | zánětlivá onemocnění | IL-1R2 |
| | podporují extravazaci buněk imunitního systému | srdeční selhání, diabetes | IL-1Ra (anakinra) |
| | | | anti-IL-1 beta mAb |
| **IL-6** | aktivace B a T lymfocytů | zvýšená hladina v séru během sepse | sIL-6R |
| | navozují horečku | RA, Crohnova choroba | anti-IL-6 Ab (siltuximab) |
| | | | anti-IL-6R-Ab (tocilizumab) |
| **INF-gamma** | antivirová aktivita | zvýšená hladina v séru během sepse | rIFN-gamma |
| **IL-12** | podporují diferenciaci TH1 lymfocytů | nádorová onemocnění | anti-IL-12 mAb |
| | navozují protinádorovou imunitní odpověď | | |

Tabulka 3. Přehled cytokinů, jejich hlavní funkce, podíl na ostatních onemocněních a jejich terapeutické využití. Zkratky: mAb – monoklonální protilátky, Ab – protilátky,

RA – revmatoidní artritida, sTNFRs – soluble TNF receptor, IL-1Ra – IL-1 receptor antagonist, rIFN-gamma – recombinant IFN-gamma, upraveno dle Schulte et al. (48).

#### 3.3.2.1. Prozánětlivé cytokiny

TNF-alfa a IL-1beta patří v patofyziologii sepse k nejvíce studovaným cytokinům. Oba cytokiny jsou velmi silné prozánětlivé mediátory a účastní se celé řady infekčních i neinfekčních (např. revmatoidní artritida a idiopatické střevní záněty) zánětlivých onemocnění (49–52). Uvolňování TNF-alfa z makrofágů začíná už během 30 minut po iniciálním impulzu, následuje transkripce genu a translace RNA, což zařazuje tento cytokin mezi časné mediátory zánětu. TNF-alfa působí přes transmembránové specifické TNF receptory (TNFR1 a TNFR2) (53). Obsazením těchto receptorů dochází k aktivaci buněk imunitního systému a uvolňování dalších imunoregulačních mediátorů. Podobně jako TNF-alfa i IL-1 beta je uvolňován především z aktivovaných makrofágů během 30 minut. Působí přes 2 typy receptorů, IL-1R1 a IL-1R2 (54,55). Jeho účinky na buňky imunitního systému jsou srovnatelné s TNF-alfa. Kromě IL-1 beta rozeznáváme ještě IL-1 alfa, kde jeho funkce není zatím úplně známa a za hlavní mediátor během sepse z rodiny proteinů IL je považován IL-1 beta.

Důležitá role TNF-alfa a IL-1 u septických stavů byla popsána v mnoha studiích na experimentálních zvířatech i u lidí. U pokusných zvířat bylo zjištěno, že injekční podání TNF-alfa způsobuje stav, který je z velké části k nerozeznání od průběhu sepse. Podobné výsledky byly publikovány i pro IL-1 beta (48). Dále bylo zjištěno, že TNF-alfa a IL-1 působí synergicky a vyvolávají šokový stav způsobený excesivní vazodilatací, zvýšenou cévní permeabilitou, plicním edémem a horečkou. Tato data podporují vedoucí roli obou cytokinů v patogenezi sepse. Podobně zde byla odhalena iniciační role bakteriálního endotoxinu. Aplikace LPS vede ke zvýšené produkci TNF-alfa a IL-1 beta a k následnému uvolňování těchto mediátorů do systémového oběhu, kdy jejich maximální koncentrace je detekována 60-90 minut po aplikaci LPS (lipopolysacharid) (56–58). Po uvolnění do systému působí oba mediátory na různých cílových buňkách, jako jsou makrofágy, neutrofily a endoteliální buňky. Zvýšená koncentrace TNF-alfa vede ke zvýšené produkci makrofágů z kmenových buněk, dále podporují aktivaci a diferenciaci makrofágů a v neposlední řadě prodlužují jejich životnost. V endotelových buňkách zvyšuje expresi adhezivních molekul (ICAM-1, VCAM-1) a chemokinů (59,60). TNF-alfa také zvyšuje přilnavost integrinu v neutrofilech a podporuje jejich extravazaci do tkání. Kromě toho tyto mediátory zesilují zánětlivou reakci, tím že aktivují makrofágy k produkci dalších prozánětlivých cytokinů (IL-6, IL-8 a MIF), kyslíkových radikálů a sloučenin dusíku (NO) (61,62).

Toto vše má za následek sepsi s orgánovou dysfunkcí. Aplikace LPS laboratorním zvířatům se proto často využívá jako standardní model sepse.

### 3.3.3. Změny farmakokinetiky léčiv podmíněné sepsí

Sepsí indukované změny hemodynamiky, metabolismu a dalších orgánových funkcí ovlivňují výrazně farmakokinetiku řady léčiv, prakticky na úrovni všech procesů. Koncentrace léčiv v plazmě mohou být sníženy bezprostředně po podání ($C_{max}$) v důsledku snížené biologické dostupnosti, pokud jsou léky podávány *per os* nebo v důsledku zvýšeného distribučního objemu. Naopak plazmatická koncentrace léčiv může být zvýšená zejména v eliminační fázi kvůli nižší vazbě na albumin, sníženému metabolismu nebo snížené renální i biliární exkreci. Ze stejného důvodu může být zvýšená plazmatická koncentrace metabolitů, což může vést ke zvýšené toxicitě (63,64). Detailní znalosti o změnách farmakokinetických procesů během sepse jsou proto velmi důležité pro adekvátní výběr a dávkování léčiv s cílem dosáhnout optimálních výsledků terapie při minimalizaci rizika toxicity.

#### 3.3.3.1. Absorpce

Změny farmakokinetiky na úrovni absorpce jsou zejména důsledkem dysfunkce v cirkulaci, doprovázející sepsi a septický šok, která vede ke snížené perfuzi svalů, kůže a orgánů ve splanchnické oblasti; negativní vliv má rovněž venostáza v GITu v důsledku zhoršeného odtoku (65). Absorpce léčiv je pomalejší, často nekompletní, proto bývá v těchto stavech preferován intravaskulární způsob aplikace. Terapie vasopresory, jako jsou norepinefrin nebo dopamin během sepse ukázala zvýšené prokrvení ve splanchnické oblasti, což může dále přispívat k variabilitě těchto dějů (66–68).

#### 3.3.3.2. Distribuce

Rychlost a rozsah distribuce léčiv jsou kromě vlastností léčiva určovány i srdečním výdejem, místním (regionálním) průtokem krve, vazebnou kapacitou proteinů, permeabilitou membrán a změnou objemu tělesných tekutin. Všechny tyto parametry se mohou v průběhu sepse a septického šoku měnit, čímž může dojít i ke změnám v distribučním objemu léčiv (69).

*Změny v perfuzi tkání:* Na zvířecím modelu sepse se ukázalo, že dochází k redistribuci krve do životně důležitých orgánů, jako je mozek a srdce na úkor prokrvování ledvin, sleziny a GITu. Po intravenózním podání léčiv během sepse byla proto v mozku a srdci často zaznamenána zvýšená koncentrace podávaného léčiva (70,71). Týká se to především vysoce

liposolubilních látek, jako jsou anestetika. Naopak distribuce do tkání se sníženým prokrvením je během sepse omezena. To lze dokumentovat na příkladu gentamicininu podaného intravenózně. U nemocných v septickém stavu byla významně snížena koncentrace antibiotika v mikrocirkulaci v porovnání s centrální cirkulací – účinek byl následně nedostatečný (69). Pro dosažení koncentrace léčiva nutné k eradikaci infekce bylo proto potřeba dávku zvýšit. To však zvyšuje riziko orgánové toxicity.

*Změny v permeabilitě membrán:* S pokračujícím rozvojem zánětu se během sepse působením prostaglandinů, leukotrienů a aktivovaným komplementem prohlubuje endoteliální poškození. V mikrocirkulaci dochází ke zvýšené propustnosti kapilár a hromadění intersticiální tekutiny, tzv. capillary leak syndrome. Tento jev může mít vliv především na distribuci léčiv s malým distribučním objemem. Jako příklad lze uvést zvýšený distribuční objem gentamicinu u septických pacientů během akutní fáze, který je přičítán zvýšené propustnosti kapilár. Kromě sníženého prokrvování tkání, jak bylo uvedeno výše, vede zvýšená propustnost kapilár ke snížení koncentrace léčiva v plazmě. Zvýšená dávka antibiotika by znamenala zvýšenou toxicitu, proto je nutno navíc prodloužit dávkovací interval, aby údolní koncentrace v plazmě dostatečně klesly (72).

*Změny ve vazbě na protein:* Změna ve vazbě léčiva na protein během sepse může být dána změnami koncentrace proteinů v plazmě. Snížená koncentrace plazmatických proteinů a tím i snížená vazba léčiva na protein během ledvinného a jaterního poškození vede k tendenci ke zvýšení intenzity účinku včetně toxicity. Během sepsí navozeného renálního selhávání byly navíc prokázány konformační změny molekuly albuminu, což může vést ke snížené vazbě léčiv kyselé povahy na tento plazmatický protein (73).

*Změny v tělesných tekutinách – složení kompartmentů:* Zadržování tekutin způsobené selháváním srdce a náhrada tekutin jako součást léčby septické šoku se podílejí na zvýšení distribučního objemu. Zadržování tekutin je také příznakem selhání ledvin, kdy dochází ke změně celkové vody v těle a tím i ke změně v distribuci mnoha léčiv (66).

#### 3.3.3.3. Renální eliminace

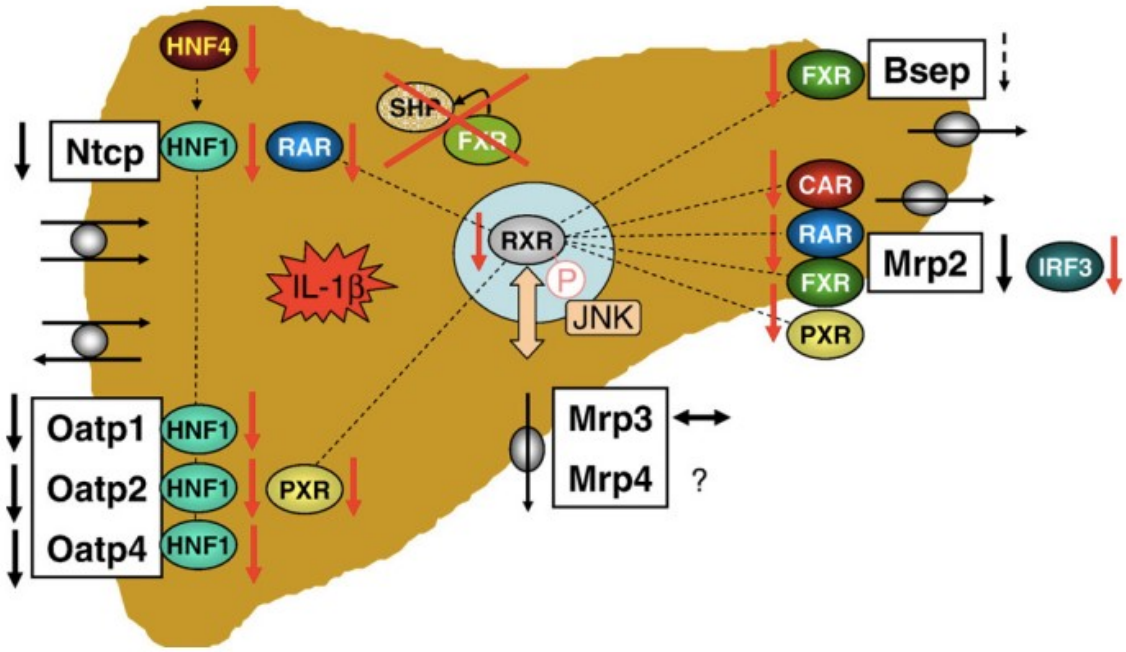
Pro většinu léčiv jsou ledviny hlavní cestou exkrece (3). Až u 15-23 % septických pacientů se rozvíjí akutní renální selhávání. Tato situace vede u léčiv s primárně renální cestou eliminace k jejich hromadění v organismu a ke zvyšování rizika toxických projevů (74).

Postiženy jsou všechny exkreční mechanismy. Snížený průtok krve se projevuje sníženou rychlostí glomerulární filtrace (GFR). Následuje ischemie tubulární části, což vede ke zhoršení vylučování endogenních látek a xenobiotik, které je dále ovlivněno změnami v expresi genů transportních proteinů v tubulech (75–78). Předchozí studie popsaly během sepse na zvířecích modelech downregulaci Oat1, Oat3 a Oct1-Oct3 transportérů na bazolaterální membráně. Naopak indukcí iNOS dochází během sepse k up-regulaci transportéru Mdr1 na apikální straně buněk tubulů, což napomáhá exkreci hromadících se toxických látek a ochraně před dalším poškozením tubulů (79,80). Celkově dochází během sepse k poklesu renální clearance léčiv a s tím souvisejícímu prodloužení biologického poločasu eliminace.

#### 3.3.3.4. Jaterní eliminace

Pro řadu léčiv jsou primárním orgánem eliminace játra. Probíhá zde nejenom biliární exkrece, ale především metabolická konverze za účelem zvýšení rozpustnosti molekuly ve vodě. Hlavními determinanty jaterní clearance jsou průtok krve játry, vazebnost léčiv v plazmě a činnost enzymů a transportérů podílejících se na eliminaci. Během septického stavu mohou být všechny tyto parametry ovlivněny. Sepse snižuje průtok krve játry, což může zvýšit zejména dostupnost léčiv s vysokým extrakčním poměrem. Většina léčiv je metabolizována v játrech enzymy CYP450. Během sepse je aktivita těchto enzymů snížená (69). Významně rovněž klesá exprese a aktivita transportních proteinů pro léčiva. Jelikož tyto transportní systémy jsou důležité i pro tvorbu žluče, je sepse nebo podání LPS spojeno s intrahepatální cholestázou (81). Tyto změny jsou důsledkem působení prozánětlivých cytokinů (především TNF-alfa a IL-1beta) i přímé LPS-TLR aktivace na hepatocytech. Změny se týkají transportérů jak na bazolaterální, tak i apikální membráně – Obr. 6. Na bazolaterální membráně se snižuje přítomnost Oatp1, Oatp2 i Ntcp přenašečů s cílem snížit vstup toxických látek typu žlučových kyselin do hepatocytů a tím bránit dalšímu rozvoji cholestázy (76,77). Downregulace exprese genu Ntcp během zánětem navozené cholestázy je způsobena sníženou vazebnou aktivitou transkripčního nukleárního faktoru HNF1 alfa a RXR:RAR. Změny v dostupnosti a vazebné aktivitě těchto dvou hlavních transaktivátorů jsou vyvolané aktivací zánětlivé intracelulární signalizační kaskády (82–84). Snížení Oatp1a1, 1a4 a 1b2 během akutní fáze je částečně způsobena sníženou expresí a aktivitou regulátoru HNF1 alfa. Kromě toho může hrát roli i exprese indukčních PXR a CAR nukleárních receptorů během akutní fáze, kdy LPS vede k jejich značné redukci (85). Na kanalikulární membráně tak dochází k redukci hlavních exportních pump pro žlučové kyseliny a řady dalších anorganických aniontů, Bsep a Mrp2 (86,87), které jsou právě regulovány FXR, RAR, PXR a CAR. Celkově tedy významným

způsobem klesá eliminace substrátů lékových transportérů, což vytváří základní podmínku pro kumulaci a rozvoj toxicity. Méně je znám vliv endotoxinové cholestázy na expresi efluxních transportérů Mrp3 a Mrp4 na bazolaterální membráně hepatocytů. U myší aplikace endotoxinu nebo léčba TNF-alfa vyvolala snížení Mrp3 mRNA, na druhou stranu u potkanů léčených LPS byl efekt opačný. Tento rozdíl může znamenat mezidruhové rozdíly, které můžeme pozorovat u zánětem navozené regulace transkripce (81).




Obr 6. Regulace transportérů při endotoxinem navozené cholestáze. Celkově dochází ke snížení exprese transportérů na bazolaterální i kanalikulární membráně hepatocytů. Hlavním regulátorem poklesu exprese Mrp2 a Ntcp je zejména IL-1 beta. Downregulace Oatp1a1, Oatp1a2 a Oatp1a4 je zprostředkovaná sníženou expresí a aktivitou HNF1 alfa. Bsep a Mrp2 je regulován sníženou expresí nukleárních receptorů FXR, PXR a CAR. Zkratky: PXR – Pregnane X Receptor, CAR - Constitutive Androstane Receptor, HNF1 alfa - Hepatocyte Nuclear Factor 1 alfa. Upraveno dle Geier A., et al. (81).

## 3.4. Léčba sepse

Terapeutické intervence se během sepse soustřeďují na udržení náplně cévního řečiště, krevního tlaku, perfúze orgánů, dále pak na potlačení infekce a případně i zánětlivé reakce.

### 3.4.1. Náhrada tekutin a vasopresory

Při sepsi je důležitá intravenózní náhrada tekutin, aby se zabránilo hypovolémii a selhávání ledvin. Při těžké sepsi nebo septickém šoku se náhradní tekutiny podávají nitrožilně během prvních 24 až 48 hodin. Nezbytné je monitorování tvorby moče, aby se mohly včasně odhalit příznaky ledvinného selhávání, které bývá častou komplikací sepse nebo septického šoku. Pokud sepse způsobí snížení krevního tlaku, používají se vasopresory. Využívá se jich především v situaci, když hypotenze nereaguje na počáteční náhradu tekutin k udržení středního arteriálního tlaku. Dle oficiálních doporučení jsou hlavními léčivy v tomto směru noradrenalin a dopamin (88). U pacientů se septickým šokem s těžkou hypotenzí a vysokým srdečním výdejem, u nichž náhrada tekutin a podání vysokých dávek konvenčních katecholaminů selhala, lze zvážit podání terlipresinu, analogu vazopresinu (89).

### 3.4.2. Antibiotika

Antibiotika jsou hlavní kauzální léčbou sepse i septického šoku. Při těžké sepsi nebo septickém šoku jsou antibiotika podávána nitrožilně (*i.v.*). V ideálním případě by měla být léčba antibiotiky zahájena co nejdříve, nejlépe do jedné hodiny od diagnózy, aby se zabránilo závažným komplikacím nebo smrti. Důležité je určit pravděpodobný zdroj infekce a podávat intravenózně širokospektré antimikrobiální látky, dokud nejsou k dispozici výsledky identifikace a rezistence, kdy se pak použijí antibiotika cíleně proti určitému typu bakterií (90). Sepse může být způsobena buď gram-negativními bakteriemi (*Enterobacteriacea, Haemophilous influenzae, Pseudomonas aeruginosa, Bacteroides fragilis, E. coli, Klebsiella* a další) nebo gram-pozitivními bakteriemi (*Staphylococcus spp., Streptococcus spp., Enterococcus spp., Clostridium spp*. a další) a v neposlední řadě dalšími mikroorganismy (*Candida spp., Mycoplasma pneumoniae, Legionella spp*., viry a další) (91).

Renální exkrece je základní eliminační cestou minimálně u 60 % antibiotik. Během sepse dochází k zásadním změnám funkce jednotlivých procesů, které mohou mít vliv jak na farmakokinetiku, tak následně na farmakodynamiku antibiotik, zejména ve smyslu jejich kumulace a potenciace toxicity. Optimálním řešením by bylo provádění terapeutického monitorování plazmatických koncentrací podaných léčiv a jejich metabolitů s následně

individualizovanou změnou dávky. Nicméně měření hladin je proces značně náročný a nelze ho zabezpečit v dostatečném rozsahu. Z toho důvodu je důležité náležitě prozkoumat charakter změn jednotlivých mechanismů zapojených do farmakokinetiky léčiv během sepse. Následně je možné predikovat farmakokinetiku konkrétních léčiv na základě znalostí jejich vlastností a zapojených farmakokinetických dějů. V tomto ohledu je rovněž důležité popsat změny zapojených procesů indukovaných současně aplikovanou léčbou. Především se jedná o účinek léčiv modulujících zánětlivou odpověď.

### 3.4.3. Glukokortikoidy

Rozsah poškození a intenzita zánětlivé odpovědi je závislá na rovnováze mezi zánětlivými a kompenzačními protizánětlivými reakcemi. Sepse, septický šok a následky systémové zánětlivé odpovědi na infekci patří mezi třináct nejčastějších úmrtí ve Spojených Státech Amerických (92). Mělo se za to, že včasná protizánětlivá léčba by mohla mít vliv na průběh sepse a septického šoku. Kortikosteroidy byly mezi prvními protizánětlivými a imunomodulačními látkami, které se testovaly u nemocných v sepsi. Studie s vysokými dávkami kortikosteroidů neprokázaly u septických pacientů zlepšení, a navíc u některých léčba vysokými dávkami vedla ke zvýšené mortalitě. V poslední době se objevil nový koncept léčby, kdy jsou pacientům podávány kortikosteroidy vícekrát, ale v nižších dávkách (hydrokortison 100 mg *i.v.* 3x denně po dobu 5 dní) (93). Výsledky randomizované studie Carlet J. et al. (94) jsou slibné a ukazují na nové využití kortikosteroidů v léčbě sepse. Potenciálně příznivý efekt glukokortikoidů během sepse je přisuzován jejich výraznému protizánětlivému působení – přehled zapojených mechanismů nabízí Tabulka 4. Pozitivně se mohou projevit i další účinky glukokortikoidů: stabilizace lysozomálních enzymů, snížení propustnosti kapilár, snížení koagulopatie, antagonizace účinků endotoxinů a snižení místní zánětlivé odpovědi a uvolnění mediátorů zánětu. Dále podporují vazokonstrikční účinky na kapilární řečiště, a to buď přímo, nebo nepřímo prostřednictvím adrenergního systému (93).

| účinek na cytokiny | účinek na neutrofily | ostatní účinky |
|---|---|---|
| downregulace cytokinů a TNF alfa | stabilizace neutrofilních lysozomů | inhibice iNOS, COX-2, $PLA_2$ |
| zabránění uvolnění TNF a IL z mononukleárních buněk | zabránění uvolňování lysozomálních enzymů | zabraňují aktivaci komplementu |

Tabulka 4. Přehled protizánětlivých účinků glukokortikoidů, Zkratky: iNOS – inducible nitric oxide synthase, COX-2 – cyklooxygenase 2, TNF – tumor necrosis factor, IL – interleukin, $PLA_2$ – phospholipase A2, upraveno podle Klaitman et al. (93).

Účinek glukokortikoidů je zprostředkován aktivací glukokortikoidního receptor (GR), který patří do skupiny nukleárních receptorů. Po navázání glukokortikoidů na glukokortikoidní receptor, který se nachází v cytoplazmě, dochází k aktivaci tohoto receptoru a k translokaci komplexu glukokortikoid – GR do jádra. Uvnitř jádra můžou glukokortikoidy regulovat genovou transkripci několika cestami: a) přes navázání komplexu GK-GR na specifickou sekvenci DNA, čímž dochází k přímé aktivaci exprese genů; b) interakcí s jinými transkripčními faktory; c) prostřednictvím modulace stability specifické mRNA molekuly (post-translační modulace) (95). Navázáním komplexu GK-GR na glukokortikoid odpovídající element (GRE) dochází k aktivaci transkripce genů kódující protizánětlivé látky, jako jsou lipokortin 1, IL-10, antagonista IL-1 receptoru a další (96).

Nejvýraznějším účinkem glukokortikoidů je inhibice exprese genů pro zánětlivé mediátory. K tomu nedochází přímou reakcí mezi GR a GRE, neboť u většiny zánětlivých mediátorů nejsou v promotorových částech genu přítomna tato GRE místa. Spíše je to přímou interakcí mezi aktivovaným GR a aktivovanými transkripčními faktory. Je to tzv. negenomová cesta (transreprese), kdy dochází k navázání transkripčních faktorů, jako nukleární faktor kappa B, aktivační protein – 1 (AP-1) a dalších. To má za následek regulaci exprese zánětlivých genů se sníženou tvorbou prozánětlivých mediátorů a cytokinů (96).

Glukokortikoidy dokážou měnit i strukturu chromatinu. Glukokortikoidní receptory interagují s vazebným proteinem pro CREB (c-AMP-response element binding protein), který působí jako kofaktor transkripce, a vážou několik dalších transkripčních faktorů, které mezi sebou konkurují o vazebná místa na této molekule (96). Translační modulace, která má za následek sníženou stabilitu mRNA a snížený biologický poločas, byla pozorována např. u IL-1beta a IL-6 (95).

### 3.4.4. Anti-cytokinová terapie

Regulace rovnováhy cytokinů, která zahrnuje prozánětlivé, protizánětlivé cytokiny, ale také inhibitory prozánětlivých cytokinů, jako jsou sTNFRs, IL-1Ra a IL-1R2, je na jedné straně důležitá pro eliminaci patogenů napadajících organismus a na druhé straně pro zmírnění poškození tkáně zánětlivou reakcí (48). Cytokinové receptory a antagonisté receptorů TNFRs, IL-1R2 a IL-1Ra modulují činnost TNF-alfa a IL-1, kdy antagonizací receptorů můžeme zmírnit jejich účinky v organismu. U dobrovolníků byly po aplikaci endotoxinu změřeny v systémovém oběhu zvýšené hladiny TNFRs a IL-1Ra. U myších modelů septického šoku podání IL-1Ra zvýšilo přežití, což potvrzuje potenciální terapeutický účinek IL-1Ra. Při měření koncentrací u septických pacientů se pro TNFRs nebo TNF více než absolutní plazmatické koncentrace využívá poměr mezi TNF-alfa a TNFRs. Z toho vyplývá, že pro průběh sepse je důležitá určitá rovnováha mezi cytokiny a jejich rozpustnými inhibitory receptorů. Přesný mechanismus stále není plně znám. Léčba anti-cytokiny nebyla doposud u kriticky nemocných pacientů se sepsí účinná, což odráží složitost patofyziologie tohoto stavu. Nejvíc nadějným se jeví IL-RA, jelikož byl připraven i syntetický analog a výsledky ukazují, že tyto látky mohou potlačit některé projevy sepse. Vliv této látky na eliminační děje nebyl doposud popsán. Proto se daná otázka dostala do centra naší pozornosti (48).

### 3.4.5. Potenciální ovlivnění imunitní odpovědi modulací hladiny železa pomocí chelátorů

Recentní studie naznačují, že dalším faktorem, který může modulovat zánětlivou odpověď během sepse je železo. Tento prvek má významnou funkci v redoxní rovnováze a hraje důležitou roli v oxidačním poškození tkání během zánětu (97). Studie potvrzují, že přítomnost bakterií nebo lipopolysacharidu v cirkulaci vede k velmi rychlé sekvestraci železa ve tkáních. Cílem je snížit dostupnost železa pro bakterie a následně tím inhibovat jejich růst. Iniciátorem sekvestrace železa je aktivace nespecifické imunity bakteriálními antigeny, zejména endotoxiny, které stimulují tvorbu cytokinů v makrofázích (98). Z nich zejména IL-6 je schopen aktivovat pSTAT3 kaskádu v hepatocytech, což následně vede ke zvýšené syntéze a vyplavení hepcidinu, hlavního hormonu snižujícího hladinu železa v séru. Podstatou je navázání hepcidinu na ferroportin, transportér pro železo, který zprostředkuje výdej železa, zejména z hepatocytů, makrofágů a enterocytů do séra. Komplex hepcidin-ferroportin je následně internalizován z membrány a degradován. Tím dochází ke snížení absorpce železa ve střevě a k jeho sekvestraci ve tkáních, zejména v játrech a buňkách nespecifické imunity. Hepcidin je navíc jednou z nejaktivnějších antimikrobiálních látek syntetizovaných

v organizmu během mikrobiální infekce a samotné jeho podání vykazuje příznivé účinky v potlačení příznaků sepse. V makrofázích sekvestrované železo pomáhá v inaktivaci baktérií zvýšením produkce reaktivních forem kyslíku a aktivací lyzozomů. Nicméně v dalších tkáních, zejména v játrech, současně tímto mechanismem přispívá kumulované železo i k orgánové toxicitě. V následných studiích bylo skutečně prokázáno, že během sepse může chelatace železa významně omezit rozvoj zánětlivé reakce i poškození tkání, což bylo dokonce spojeno s lepším přežíváním (99,100). Nicméně pro další využití tohoto přístupu prozatím neexistuje dostatek dat, včetně absence informací o vlivu daných látek na eliminační mechanismy pro léčiva.

### 3.4.6. Boldin

Boldin je hlavní alkaloid z kůry a listů stromu boldovníku vonného (*Peumus boldus* Molina, Monimiaceae). Tato látka je považována za nositele účinků, pro které je extrakt z boldovníku vonného používán v tradiční medicíně k léčbě bolestí hlavy, kloubů, menstruačních bolestí, dyspepsie, zánětů močového ústrojí a tvrdí se taky, že má sedativní a mírně hypnotické účinky. Boldin je proto v současnosti studován na příslušných *in vitro* a *in vivo* modelech a výsledky skutečně ukazují na jeho silné antioxidační schopnosti, které umožňují prevenci patologických stavů souvisejících s oxidačním stresem, jakými jsou poškození jater (101,102), rozvoj systémové zánětlivé reakce (103), proliferace nádorových buněk, zvýšená agregace krevních destiček nebo ateroskleróza (104). Boldin taky prokázal další účinky nesouvisející s oxidačním stresem, a to relaxační aktivitu na hladké svalstvo střev a cév krevního řečiště (pravděpodobně prostřednictvím antagonismu nikotinových a $\alpha_1$ receptorů a $Ca^{2+}$ kanálů), neuroleptický/antinociceptivní účinek (pravděpodobně díky blokádě $D_2$ receptorů) a cholagogní/choleretický účinek. Právě choleretický efekt představuje v současnosti jednu z hlavních oblastí podávání potravinových doplňků obsahujících boldin. Několik prací demonstrovalo, že *p.o.* podání extraktu boldovníku vonného může zvýšit tok žluči u potkanů i myší (105–107). Novější studie naopak nepotvrdily choleretický účinek u potkanů ani po p.o. podání 200-800 mg/kg etanolového extraktu boldovníku vonného ani po *i.v.* podání buď extraktu (4:1) nebo čistého boldinu (101). Tyto nesrovnalosti ve výsledcích ukazují na potřebu lepší charakterizace mechanismu a podmínek předpokládané cholerézy po podání boldinu. Současně není známo, jak boldin ovlivňuje cholestatické stavy.

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# 5. Cíle práce

Během sepse dochází ke změnám procesů, zejména na úrovni eliminace, které mohou výrazně modifikovat farmakokinetiku léčiv. Aby se zabránilo možným toxicitám nebo selhání efektu, je vhodné dotčené mechanismy studovat včetně potenciálního vlivu použité protizánětlivé terapie. Nedostatek informací v dané oblasti byl hlavním důvodem k formulování následujících cílů práce:

1) charakterizovat změny exprese lékových transportérů v ledvinách a v játrech během modelového septického stavu a popsat jejich funkční dopady na farmakokinetiku léčiv. V naší studii byl jako modelový substrát pro lékové transportéry použit azitromycin.
2) prostudovat vliv dvou silných protizánětlivých látek na akutní ledvinné selhání a asociované změny eliminačních mechanismů během sepse. Použity byly:
   a) anakinra – antagonista na IL-1 receptoru
   b) dexametazon – glukokortikoid
3) sledovat vliv dvou různých klinicky dostupných chelátorů železa, dexrazoxanu a deferoxaminu, jako prevence akutního poškození jater během modelového septického stavu.
4) zhodnotit vliv boldinu na mechanismy tvorby žluče a popsat efekt této látky na modelech intrahepatální cholestázy.

## 6. Seznam prací a podíl kandidátky na jednotlivých publikacích

Tato dizertační práce je předkládána jako komentovaný soubor 4 prací publikovaných v časopisech s impakt-faktorem. Všechny manuskripty jsou původní experimentální práce zaměřené na farmakokinetiku léčiv za septických stavů nebo farmakokinetiku léčiv během cholestázy, jako možný následek sepse. Kandidátka je první autorkou dvou prací (6.1 a 6.3) a spoluautorkou dalších dvou manuskriptů (6.2 a 6.4).

Podíl předkladatelky na jednotlivých publikacích je následující:

6.1. IL-1 receptor blockade alleviates endotoxin-mediated impairment of renal drug excretory functions in rats.

- praktické provedení *in vivo* studií

- 80% *in vitro* studií (PCR, WB, stanovení TBA, ELISA)

- histologie (focení a vyhodnocování)

- analýza dat a sepisování manuskriptu

6.2. Deferoxamine but not dexrazoxane alleviates liver injury induced by endotoxemia in rats.

- účast na *in vivo* studiích

- částečné provedení *in vitro* experimentů (PCR, WB, TBA)

- podíl na analýze dat a sepisování manuskriptu

6.3. Boldine enhances bile production in rats via osmotic and farnesoid X receptor dependent mechanisms.

- účast na *in vivo* studiích

- částečné provedení *in vitro* experimentů (PCR, WB, TBA)

- podíl na analýze dat a sepisování manuskriptu

6.4. Boldine attenuates cholestasis associated with nonalcoholic fatty liver disease in hereditary hypertriglyceridemic rats fed by high-sucrose diet.

- účast na *in vivo* studiích

- částečné provedení *in vitro* experimentů (PCR, WB, TBA)

- podíl na analýze dat a sepisování manuskriptu

# 7. Komentáře k jednotlivým publikacím

## 7.1. IL-1 receptor blockade alleviates endotoxin-mediated impairment of renal drug excretory functions in rats.

Kadova Z, Dolezelova E, Cermanova J, Hroch M, Laho T, Muchova L, Staud F, Vitek L, Mokry J, Chladek J, Havlinova Z, Holecek M, Micuda S

Am J Physiol Renal Physiol. 2015 Mar 1;308(5):F388-99

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Dexametazon a anakinra (antagonista na IL-1 receptorech) patří mezi silné protizánětlivé látky. Hlavním cílem bylo zjistit, zda mohou mít tyto látky vliv na akutní ledvinné poškození a s tím spojené ovlivnění exkrečních funkcí během endotoxémie u potkanů.

Deset hodin po aplikaci LPS jsme u neléčených endotoxemických potkanů mohli pozorovat typické symptomy akutního poškození ledvin, jako jsou snížená glomerulární filtrace, změněná tubulární exkrece močoviny a sodíku a snížená ledvinná exkrece azitromycinu (substrátu pro Mrp2 a Mdr1, tj. hlavních lékových efluxních transportérů). Po aplikaci obou látek došlo ke zmírnění zánětlivých odpovědí, poškození jater a akutního ledvinného selhávání. Zvýšila se renální clearance azitromycinu obnovením glomerulární filtrace, bez výrazných změn na jeho tubulární sekreci. Současně jsme pozorovali rozdílný účinek obou léčiv na renální expresi jednotlivých transportérů. Pouze po aplikaci dexametazonu došlo ke zvýšení renální clearance žlučových kyselin, jako reakci na redukci transportéru pro jejich tubulární reabsorpci Asbt. Obě látky příznivě tlumily cholestázu navozenou LPS v játrech.

Souhrnně můžeme říci, že naše data u obou látek prokázala jejich účinnost v prevenci proti akutnímu ledvinnému poškození vzniklému během endotoxemie. Došlo k obnovení renálního vylučování léčiv, především díky zlepšení glomerulární filtrace. Vliv obou léčiv na tubulární funkce a exkreci léčiv byl rozdílný, s důrazem na nutnou znalost transportní cesty pro individuální léčiva používaná během sepse. Účinek anakinry dále potvrzuje významný podíl IL-1 v patogenezi endotoxinem (LPS) navozeného akutního ledvinného poškození.

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# IL-1 receptor blockade alleviates endotoxin-mediated impairment of renal drug excretory functions in rats

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**Kadova Z, Dolezelova E, Cermanova J, Hroch M, Laho T, Muchova L, Staud F, Vitek L, Mokry J, Chladek J, Havlinova Z, Holecek M, Micuda S.** IL-1 receptor blockade alleviates endotoxin-mediated impairment of renal drug excretory functions in rats. *Am J Physiol Renal Physiol* 308: F388–F399, 2015. First published December 10, 2014; doi:10.1152/ajprenal.00266.2014.—The aim of our study was to investigate whether two potent anti-inflammatory agents, dexamethasone and anakinra, an IL-1 receptor antagonist, may influence acute kidney injury (AKI) and associated drug excretory functions during endotoxemia (LPS) in rats. Ten hours after LPS administration, untreated endotoxemic rats developed typical symptoms of AKI, with reduced GFR, impaired tubular excretion of urea and sodium, and decreased urinary excretion of azithromycin, an anionic substrate for multidrug resistance-transporting proteins. Administration of both immunosuppressants attenuated the inflammatory response, liver damage, AKI, and increased renal clearance of azithromycin mainly by restoration of GFR, without significant influence on its tubular secretion. The lack of such an effect was related to the differential effect of both agents on the renal expression of individual drug transporters. Only dexamethasone increased the urinary clearance of bile acids, in accordance with the reduction of the apical transporter (Asbt) for their tubular reabsorption. In summary, our data demonstrated the potency of both agents used for the prevention of AKI, imposed by endotoxins, and for the restoration of renal drug elimination, mainly by the improvement of GFR. The influence of both drugs on altered tubular functions and the expression of drug transporters was differential, emphasizing the necessity of knowledge of transporting pathways for individual drugs applied during sepsis. The effect of anakinra suggests a significant contribution of IL-1 signaling to the pathogenesis of LPS-induced AKI.

dexamethasone; anakinra; endotoxins; drug transporters; acute kidney injury

THE ENDOTOXINS RELEASED FROM the cell wall of gram-negative microorganisms play an important role in the initiation and maintenance of the severe inflammatory response of organisms during sepsis. These lipopolysaccharides bind to toll-like receptors (TLR) in tissues, especially at the surface of macrophages, and trigger a nuclear factor-κB-directed immune response. This response is based on the increased production of proinflammatory cytokines, such as TNF-α, IL-1β, and IL-6, and nitric oxide (NO) released by inducible nitric oxide synthase (iNOS), which subsequently distort the morphology and function of several organs (6). Untoward acute injury can be seen especially in the kidney (acute kidney injury; AKI) and typically includes reduced blood flow, with a decreased glomerular filtration rate (GFR). Consequent ischemia of tubular segments leads to an alteration of transport functions, with reduced urinary concentration ability and eventually to acute tubular necrosis. The urinary excretion of endo- and xenobiotics is further mitigated by profound changes in the expression of transporting proteins in tubules (2, 5, 18, 26, 27). In this situation, the pharmacological modulation of the regulatory mediator pathways, such as the inhibition of iNOS or activation of the protective heme oxygenase (HMOX)-CO pathway, may significantly restore GFR and tubular excretory functions (20, 35) and consequently affect the pharmacokinetics of drugs administered in therapy for sepsis (55). However, the influence of immune response-modifying agents on the drug-excretory capacity of the renal tubule during such a status has not been described as yet.

The drugs which have a significant immunosuppressive effect, which are routinely administered to patients during septic shock, are glucocorticoids (29). The rationale for their use is to support the insufficient endogenous secretion of cortisol, to enable the maintenance of the vascular reactivity to catecholamines, and consequently, blood pressure. However, corticosteroids in applied dosages also possess a strong genomic effect, based on the suppression of NF-κB and their

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target proinflammatory cytokines (7). As a consequence, with sepsis, corticosteroids terminate systemic and tissue inflammation, restore organ function, and prevent death (3). In the kidney, the protective corticosteroid effect includes the inhibition of iNOS activity, the prevention of hypoxic injury to the cortex, the improved permeability of the glomerular endothelium, and the normalization of water clearance (25, 40, 47). In addition, corticosteroids are known for their ability to induce drug transporters in the liver during endotoxemia (6a). Taken together, these drugs may have strong potential to modify renal excretion of therapeutics and endobiotics during sepsis.

An alternative therapeutic strategy for the improvement of survival, as well as for the prevention of acute tubular injury during endotoxemia or sepsis, may be the more specific anticytokine approach using IL-1 receptor blockade (4, 11–13, 36). Although later studies were unable to find a positive effect of a recombinant human IL-1 receptor antagonist on septic acute renal failure (52), the synthetic IL-1 receptor antagonist anakinra showed a preventive effect on endotoxin-induced downregulation of some crucial drug transporters in the liver (16). Corresponding data about a renal effect are missing. The objective of the present study was, therefore, to investigate the influence of both the corticosteroid dexamethasone and anakinra on the renal excretory pathways for endo- and xenobiotics in the rat model of endotoxin-induced AKI.

## MATERIALS AND METHODS

*Chemicals.* Azithromycin (AZT) was used in its original formulation for parenteral administration (Sumamed, Pliva-Lachema, Brno, Czech Republic); dexamethasone was purchased from Sigma (St. Louis, MO); ANA was used in its original formulation for parenteral administration (Kineret, Biovitrum, Stockholm, Sweden), and LPS (from *Salmonella enterica* serotype typhimurium) was obtained from Sigma-Aldrich (St. Louis, MO).

*Animal models.* All procedures were approved by the Animal Care Committee at Charles University in Prague, Faculty of Medicine in Hradec Kralove. Male Wistar rats (280–320 g; purchased from Konarovice, Czech Republic) were kept under temperature- and light-controlled conditions, on a 12:12-h light-dark cycle. Throughout the study, the rats were fed a commercial food diet and had free access to water. The rats were divided into the following four groups ($n = 12$ for each): *1*) control group, which received only saline at designated time points; *2*) LPS-treated group, which was injected with 4 mg/kg body wt of LPS intraperitoneally (ip); *3*) DEX+LPS-treated group, which received 10 mg/kg body wt of dexamethasone ip 1 h before LPS administration; and *4*) ANA+LPS-treated group, which received 10 mg/kg body wt of anakinra ip 1 h before and 4 h after LPS treatment. The regimens were selected on the basis of previous studies (6a, 16). The administration of a steroid before LPS in rodents is commonly used because of the reduced receptor-binding capacity for glucocorticoids under septic conditions in animals (9, 44). Anakinra was used with the same regimen as recommended previously (16) only we have added the dose 5 h after initial administration due to the short half-life of the agents in rats. After the application of LPS or corresponding saline, all the animals were housed in metabolic cages for 10 h, where water consumption was monitored and urine was collected. Thereafter, the animals were anesthetized ip with pentobarbital sodium (50 mg/kg). Six animals from each group were euthanized by exsanguination to obtain plasma for biochemical analysis and the liver and kidneys for expression analysis; samples were snap-frozen in liquid nitrogen and stored at −80°C until analysis. The other six animals from each group underwent an in vivo clearance study.

*In vivo clearance study.* Rats were under pentobarbital sodium anesthesia (50 mg/kg ip). The right jugular vein used for drug administration and the continuous infusion of physiological saline (2 ml/h), the left carotid artery for blood sampling, the common bile duct for bile collection, and the urinary bladder for urine collection were cannulated. Thereafter, the rats received a single bolus of AZT (20 mg/kg). Blood samples (~0.3 ml) were taken at designated time intervals (0, 4, 10, 30, 60, 120, 180, and 240 min) after the injection of AZT. Bile and urine samples were collected in preweighed tubes at 30-min intervals throughout the experiment. Blood was centrifuged at 2,000 *g* for 5 min at 4°C to obtain plasma. Organs were snap-frozen in liquid nitrogen and, together with plasma, bile, and urine samples, kept at −80°C until assayed.

*Analytic procedures.* The concentrations of AZT, citrulline/arginine, and nitrites/nitrates were determined by previously described HPLC methods (14). Biochemical analyses of serum and urine were performed on a Cobas Integra 800 and Cobas Mira Plus (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's instructions. Bile acids were determined from serum, urine, and bile using a commercial kit (Diazyme Europe) according to the manufacturer's instructions. Fractional excretion was calculated according to the following formulas: $FE_{Na} = 100 \times$ (urine sodium $\times$ plasma creatinine)/(plasma sodium $\times$ urine creatinine); $FE_{urea} = 100 \times$ (urine urea $\times$ plasma creatinine)/(plasma urea $\times$ urine creatinine).

*Pharmacokinetic analysis.* Data were analyzed with Kinetica and MS Excel software. Pharmacokinetic parameters of intravenously administered AZT were calculated according to standard noncompartmental techniques (14). The area under the plasma concentration-time curve ($AUC_{0-\infty}$) was calculated from the sum of the $AUC_{0\text{-}Tlast}$, estimated by the log-linear trapezoidal rule from *time 0* to the time of the last measured concentration ($T_{last}$), and the AUC was extrapolated to infinity ($AUC_{Tlast\text{-}\infty}$) and calculated as the last measured concentration divided by the elimination constant ($K_{el}$), estimated on the basis of linear regression analysis. The half-life ($T_{1/2}$) was calculated by dividing ln(2) by $K_{el}$. Total clearance ($CL_{Tot}$) was calculated as $CL_{Tot} = Dose/AUC_{0-\infty}$. The apparent volume of distribution (Vd) was calculated as $Vd = CL_{Tot} \times AUMC/AUC_{0-\infty}$, where AUMC is the area under the first moment plasma concentration vs. time curve. The biliary ($CL_{Bile}$) and renal ($CL_R$) clearance were calculated as $CL_{Bile} = X_{Bile}/AUC_{0\text{-}Tlast}$ and $CL_R = X_{Urine}/AUC_{0\text{-}Tlast}$, where $X_{bile}$ and $X_{urine}$ were the amounts of AZT excreted to bile and urine, respectively, during the evaluated period, and $T_{last}$ was 240 min. Tubular secretory clearance ($CL_{RS}$) of AZT was calculated as the difference between $CL_R$ (AZT renal clearance) − $CL_{CR}$ (creatinine clearance).

*Histological examination.* The kidneys were fixed in 10% neutral buffered formalin solution and thereafter embedded in paraffin and stained with hematoxylin-eosin. Pictures were taken using an Olympus BX51 microscope with an Olympus DP71 camera. Glomerular and tubular damage was evaluated by the same specialist.

*Western blot analysis.* One hundred milligrams of kidney or liver tissue was minced with scissors in 0.5 ml RIPA buffer (Sigma) in the presence of protease inhibitors, homogenized with ULTRA-TURRAX ($2 \times 15$ s), and centrifuged (3,000 rpm for 10 min, 4°C). Protein concentrations were measured with a BCA Protein Assay Kit (ThermoScientific), using bovine serum albumin as a standard. Proteins (100 μg) were separated on SDS-polyacrylamide gel and transferred to a polyvinylidene difluoride membrane. After blocking in Tris-buffered saline, supplemented with 0.05% of Tween 20 and 5% of nonfat dry milk, the membranes were incubated for 1 h with appropriate primary antibodies (Table 1). Blots were then incubated for 1 h in horseradish peroxidase-conjugated secondary antibodies (GE Healthcare, Prague), and the membranes were visualized with enhanced chemiluminescence reagents (ThermoPierce). The band intensities were detected using ScanMaker i900 (UMAX) and QuantityOne imaging software (Bio-Rad). Equal protein loading was confirmed by immunodetection of GAPDH (kidneys) or β-actin (liver).

Table 1. *Antibodies used in Western blotting*

| | | Primary Antibody | | Secondary Antibody | |
|---|---|---|---|---|---|
| Protein | Source | Catalog no. | | Dilution | Detected Molecular Weight, kDa |
| Oat1 | Sigma | SAB2102179 | 1:1,000 | 1:2,000 | 62 |
| Oat2 | Sigma | AV42708 | 1:2,500 | 1:3,000 | 60 |
| Oat3 | Sigma | SAB2102179 | 1:1,000 | 1:2,000 | 62 |
| P-gp | Sigma | P7965 | 1:500 | 1:1,000 | 145 |
| Mrp2 | Alexis | ALX 801-037 | 1:500 | 1:1,000 | 190 |
| β-Actin | Sigma | A 5316 | 1:5,000 | 1:8,000 | 42 |
| Gapdh | Sigma | G 8795 | 1:5,000 | 1:8,000 | 37 |
| HMOX1 | Sigma | H4535 | 1:1,000 | 1:3,000 | 32 |
| iNOS | BD Bioscience | 610329 | 1:1,000 | 1:2,000 | 130 |

Antibodies were obtained from GE Healthcare Life Sciences: ECL mouse IgG, horseradish peroxidase (HRP)-linked anti-mouse and anti-rabbit secondary linked F (ab')$_2$ fragment (catalog no. NA931) and ECL rabbit IgG, HRP-linked F (ab')$_2$ fragment (catalog no. NA 934). HMOX1, heme oxygenase-1; iNOS; inducible nitric oxide synthase.

*Real-time qRT-PCR.* Real-time PCR was performed on a 7500HT Fast Real-Time PCR System (Applied Biosystems, Foster City, CA). Total RNA was isolated from liver and kidney tissue samples using TRIzol reagent (Sigma-Aldrich) and converted into cDNA by a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) according to the manufacturer's protocol. Thirty nanograms of cDNA was loaded into one reaction. All experiments were performed in triplicate. Genes were amplified using TaqMan Fast Universal PCR Master Mix and predesigned TaqMan Gene Expression Assay kits (Table 2). All data were normalized to GAPDH, and differences in gene expression were calculated from detected dCt values, as described previously (22).

*Detection of HMOX1 activity.* Twenty microliters of tissue sonicate was incubated for 15 min at 37°C in septum-sealed, CO-free 2-ml vials, containing 20 μl of 50 μM methemalbumin as a substrate in the absence (blank) or presence of 20 μl of 4.5 mM NADPH. Blank reaction vials contained 0.1 M potassium phosphate (pH 7.4) in place of NADPH. The amount of CO generated by the reaction and released into the vial headspace was quantified by gas chromatography. HMOX activity was calculated by the number of picomoles of CO produced per milligram of protein per hour.

*Data analysis.* We detected maintenance of mRNA expression of selected transporters during the in vivo clearance study, i.e., between 10 and 14 h after LPS challenge, despite the previously described progressive decrease in Oat1, Oat3, and Mrp2 mRNAs until 12 h after LPS administration (20, 23). This observation complies with previous reports that pentobarbital anesthesia (53) and fluid replacements (10) may modify ongoing endotoxemic injury. Therefore, we decided to use data from the 10-h time point uninfluenced by procedures of the in vivo clearance study as a reference to evaluate effect of both agents on molecular and histological parameters of AKI. Data are expressed as means ± SD. All data were analyzed using one-way ANOVA, followed by a post hoc test (Neuman-Keuls test). Analysis was performed using GraphPad Prism 5.0 software (GraphPad Software, San Diego, CA). A difference of $P < 0.05$ was considered statistically significant.

Table 2. *Predesigned TaqMan Gene Expression Assay kits (Life Technologies) used for quantitative real-time RT-PCR*

| Gene | Protein Symbol | Assay ID |
|---|---|---|
| *Abcb11* | Bsep | Rn00582179_m1 |
| *Abcc2* | Mrp2 | Rn00563231_m1 |
| *Abcc3* | Mrp3 | Rn00589786_m1 |
| *Abcc4* | Mrp4 | Rn01465702_m1 |
| *Abcb1a* | Mdr1a | Rn00591394_m1 |
| *Abcb1b* | Mdr1b | Rn00561753_m1 |
| *Slc10a1* | Ntcp | Rn00566894_m1 |
| *Slco1a2* | Oatp1a2 | Rn00756233_m1 |
| *Slc10a2* | Asbt | Rn01482843_m1 |
| *Slc22a6* | Oat1 | Rn00568143_m1 |
| *Slc22a7* | Oat2 | Rn00585513_m1 |
| *Slc22a8* | Oat3 | Rn00580082_m1 |
| *Slc22a1* | Oct1 | Rn00562250_m1 |
| *Slc22a2* | Oct2 | Rn00580893_m1 |
| *Slc22a3* | Oct3 | Rn00570264_m1 |
| *Slc47a1* | Mate1 | Rn01460731_m1 |
| *Slc47a2* | Mate2 | Rn02601013_m1 |
| *Il-6* | IL-6 | Rn99999011_m1 |
| *Tnf* | Tnf-α | Rn99999017_m1 |
| *Gapdh* | Gapdh | Rn01775763_g1 |

## RESULTS

*Effect of immunosuppressive therapy on LPS-mediated AKI.* The administration of LPS raised serum parameters typical of AKI, urea and creatinine (Table 3). The inclusion of corresponding urine analyses showed a significant decrease in creatinine clearance and an increase in microalbuminuria, the indicators of impaired glomerular filtration (GFR), which was combined with altered tubular functions, as demonstrated by changes in the fractional excretion of sodium and urea (Fig. 1). Urine production in LPS animals remained unchanged, presumably due to opposite changes between GFR and sodium fractional excretion. Pretreatment with either drug prevented the development of glomerular impairment in particular (Fig. 1, *C* and *D*). Tubular functions were mainly influenced by dexamethasone. The drug further increased an LPS-induced rise in the fractional excretion of sodium, with a consequent increase in urine production (Fig. 1), while the fractional excretion of urea was restored, which suggests its positive influence on organic anion transport.

Biochemical analyses of serum also confirmed the cholestatic potential of LPS, as indicated by increased levels of bilirubin, bile acids, and activities of ALP (Tables 3 and 4). Both agents protected against the development of all these alterations. Moreover, kinetic parameters of bile acid elimination (Table 4) demonstrated the restoration of their impaired biliary clearance, suggesting significant modification of responsible transporting proteins (see below). Dexamethasone also prevented impairment of urinary clearance of bile acids and predictably raised the level of glycemia.

Table 3. *Selected serum biochemical parameters related to endotoxemic liver and kidney impairment*

| | Ctrl | LPS | DEX-LPS | ANA-LPS |
|---|---|---|---|---|
| Conjugated bilirubin, μM | BD | 3.0 ± 1.6 | BD | BD |
| Total bilirubin, μM | 2.5 ± 0.5 | 9.3 ± 8.3* | 3.0 ± 0.6† | 3.8 ± 1.7† |
| AST, μkat/l | 2.2 ± 0.4 | 4.5 ± 1.0 | 4.1 ± 1.1 | 3.0 ± 0.3 |
| ALT, μkat/l | 0.6 ± 0.03 | 2.8 ± 0.6** | 0.7 ± 0.05†† | 1.4 ± 0.3†† |
| ALP, μkat/l | 1.7 ± 0.1 | 5.6 ± 1.7*** | 2.5 ± 0.4††† | 3.3 ± 0.6††† |
| Glucose, mM | 8.1 ± 1.3 | 5.6 ± 1.2 | 11 ± 3.2†† | 8.3 ± 3.3 |
| Urea, mM | 6.8 ± 0.7 | 21 ± 8.0*** | 11 ± 4.6†† | 13 ± 4.6†† |
| Creatinine, μM | 30 ± 6.5 | 61 ± 16.1*** | 47 ± 11*† | 44 ± 9.7*† |

Values are means ± SD; $n = 6$/group. Ctrl, control; LPS, endotoxemic rats; DEX, dexamethasone-pretreated rats; ANA, anakinra-pretreated rats; BD, below detection limit; AST, aspartate aminotransferase; ALT, alanine aminotransferase; ALP, alkaline phosphatase. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, significantly different from control values. †$P < 0.05$, ††$P < 0.01$, †††$P < 0.001$, significantly different from LPS values.

Histological evaluation showed normal architecture of renal parenchyma in control animals. LPS administration produced cellular vacuolization and impairment of apical membrane integrity in renal tubular cells, without microscopic changes in glomeruli (Fig. 2*B*). Despite variable results from tubular functional parameters (Fig. 1, *E* and *F*), both drugs were able to attenuate such pathological changes, as exemplified in Fig. 2, *C* and *D*.

*Immunosuppressive agents modulated LPS-induced changes in renal elimination of AZT.* To evaluate the function of drug-transporting proteins, we measured pharmacokinetic parameters of biliary and urinary elimination of AZT, a typical substrate for multidrug transporters Mdr1 and Mrp2 (45) (Table 5). Untreated endotoxemic animals showed significant decreases in both biliary and renal excretion and clearance of the antibiotic, which was associated with prolongation of its half-life. In the case of the liver, the change could only be attributed to the consequence of reduced active biliary secretion. Analysis of urinary parameters suggested that reduced GFR is responsible for the alteration in particular. Pretreatment with either drug did not show any consistent effect on AZT biliary clearance, but both raised renal clearance through an

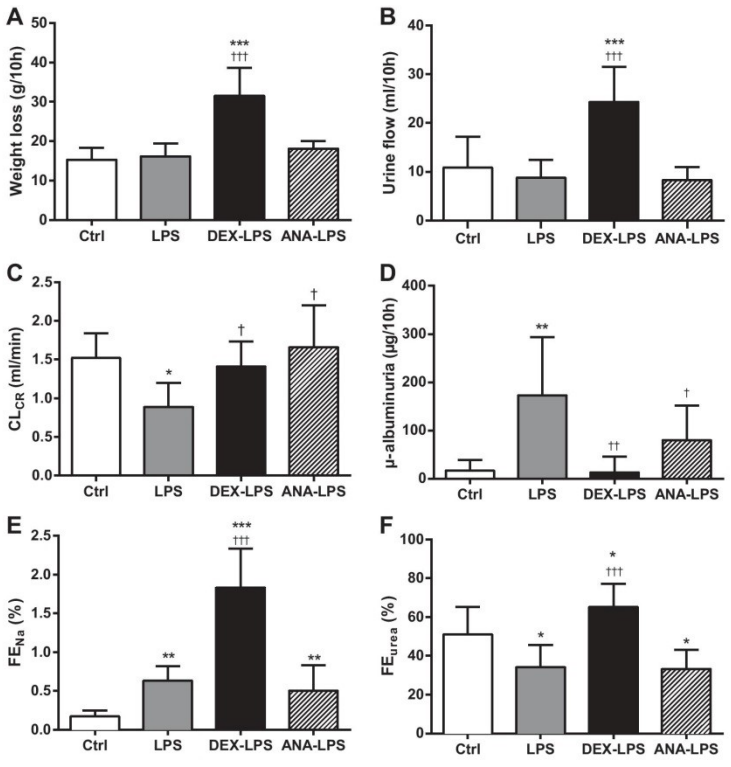


Fig. 1. Effect of dexamethasone (DEX) and anakinra (ANA) on LPS-induced changes in weight loss (*A*), urine production (*B*), creatinine clearance (*C*), microalbuminuria (*D*), and fractional excretion of sodium ($Fe_{Na}$; *E*) and urea ($FE_{urea}$; *F*). Values are means ± SD ($n = 6$). *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, compared with controls, †$P < 0.05$, ††$P < 0.01$, †††$P < 0.001$, compared with LPS.

Table 4. *Serum concentrations and excretory characteristics of bile acids*

| | Ctrl | LPS | DEX-LPS | ANA-LPS |
|---|---|---|---|---|
| Plasma TBA, μM | 17 ± 8.1 | 40 ± 19* | 20 ± 4.9† | 21 ± 11† |
| TBA $CL_{Bile}$, ml/min | 16 ± 1.6 | 4.6 ± 1.4** | 12 ± 1.8† | 13 ± 2.8† |
| TBA $CL_{Urine}$, μl/min | 1.5 ± 0.7 | 0.5 ± 0.2* | 1.3 ± 0.8† | 0.4 ± 0.2* |

Values are means ± SD; $n = 6$/group. Samples were taken at intervals of 0–30 min from the in vivo clearance study (10 h after LPS administration). TBA $CL_{Bile}/CL_{Urine}$, biliary/urinary clearance of total bile acids. *$P < 0.05$, **$P < 0.01$, significantly different from control values. †$P < 0.05$, significantly different from LPS values.

increase in GFR, which may contribute to shortening of $T_{1/2}$. Interestingly, the tendency for the reduction of Vd after LPS, most probably due to tissue hypoperfusion, becomes significant during coadministration with dexamethasone. The effect may reflect the reduced distribution of AZT into tissues, as a consequence of restored blood vessel permeability after the administration of an immunosuppressant during endotoxemia. In agreement, total clearance of AZT remained limited in the dexamethasone group, despite an increase in renal clearance.

*LPS-induced changes in renal drug transporters were differentially changed by applied immunosuppressants.* Important renal transporters, which are responsible for the elimination of AZT and other organic anions and drugs used in the treatment of sepsis, were determined. LPS treatment significantly upregulated mRNA levels of transporters at the apical membrane (Mrp2, Mrp4, Mdr1b, and Asbt) and downregulated basolateral Oat and Oct uptake transporters, as well as the multidrug and toxin extrusion protein 1 (Mate1) apical transporter, compared with controls (Table 6). The administration of either drug prevented changes in apical membrane transporters, where dexamethasone also showed a protective effect on Asbt and Bsep However, both only had a minimal effect on the transcription of basolateral molecules. In addition, we also analyzed mRNA expression of genes crucial for AZT pharmacokinetics in samples from the in vivo clearance study, i.e., 14 h after LPS challenge (Table 7). The administration of both agents demonstrated similar effect as at 10 h.

With respect to the anionic nature of AZT, we further analyzed the protein expression of Oat1-3, Mrp2, and P-gp (Fig. 3). Using crude membrane fractions, we confirmed the transcriptional downregulation of Oat1 and the upregulation of Mrp2 in untreated LPS rats. Oat2 and Oat3 proteins were posttranscriptionally upregulated by endotoxins. The administered drugs had diverse effects on transporters; while dexamethasone prevented Mrp2 induction, anakinra restored Oat1 and Oat2 levels.

To confirm the reliability of our model, we also evaluated the influence of LPS on mRNA expression of corresponding hepatic transporters. The majority of the changes observed in our study, i.e., the upregulation of Mdr1b and the downregulation of other bile acid or drug transporters in untreated LPS rats (Table 8), complies with former reports (6a, 16, 17). Only dexamethasone was able to partially alleviate changes in Ntcp, Bsep, and Mdr1b gene transcription, while anakinra accentuated those in Mrp4 and Mdr1b (Table 8).

*Effect of immunosuppressive therapy on LPS-mediated changes in regulatory molecules.* We evaluated the effect of both agents on two principal pathways regulating renal damage during endotoxemia, CO and NO. LPS treatment significantly enhanced HMOX1 expression and CO production in both the liver and kidneys (Fig. 4). Dexamethasone, as well as anakinra, returned both indicators to the control level. Similarly, untreated LPS showed a marked induction of iNOS in both organs, followed by an increase in NO plasma concentrations (Fig. 5). Only dexamethasone was able to significantly reduce the tissue content of the iNOS protein, which corresponded to

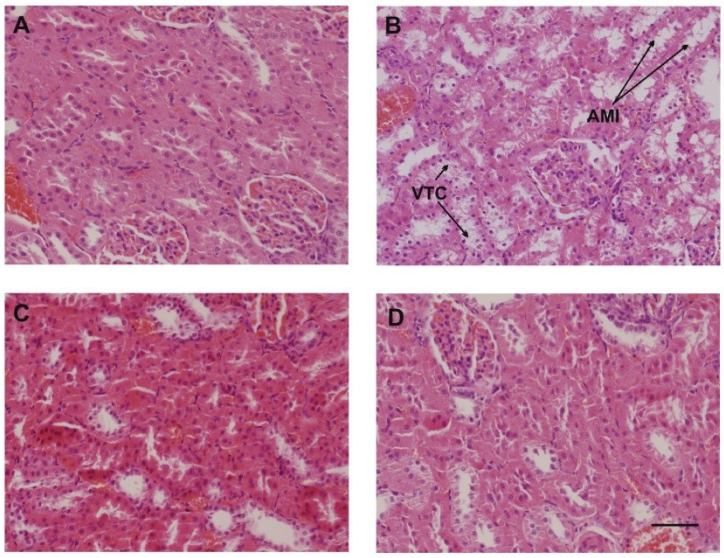


Fig. 2. Effect of immunosuppressive agents on histological changes induced by endotoxins in kidneys. Administration of both DEX and ANA was able to prevent damage to apical membrane integrity (AMI), and vacuolization of tubular cells (VTC), seen in LPS-treated animals. Representative histological slides are shown. *A*: control. *B*: LPS-administered. *C* and *D*: DEX (*C*)- or ANA (*D*)-pretreated LPS rats (bar = 100 μm).

Table 5. *Pharmacokinetic parameters of intravenously administered azithromycin (20 mg/kg)*

| | Ctrl | LPS | DEX-LPS | ANA-LPS |
|---|---|---|---|---|
| $Vd_{ss}$, l/kg | 9.4 ± 4.1 | 6.9 ± 2.4 | 3.8 ± 1.6*† | 6.2 ± 2.8 |
| $AUC_{0-\infty}$, mg·l$^{-1}$·min$^{-1}$ | 377 ± 80 | 552 ± 153 | 627 ± 187 | 360 ± 225 |
| $T_{1/2}$, min | 148 ± 62 | 232 ± 76* | 120 ± 62† | 89 ± 32†† |
| $X_{Bile}$, mg/240 min | 0.15 ± 0.03 | 0.08 ± 0.03** | 0.11 ± 0.03† | 0.1 ± 0.04 |
| $X_{Urine}$, mg/240 min | 1.1 ± 0.3 | 0.6 ± 0.3* | 1.2 ± 0.2†† | 0.9 ± 0.2 |
| $CL_{Total}$, ml/min | 21 ± 3.6 | 14 ± 3.7* | 13 ± 3.6* | 20 ± 6.9 |
| $CL_{Bile}$, ml/min | 0.5 ± 0.08 | 0.2 ± 0.1* | 0.2 ± 0.06* | 0.4 ± 0.2 |
| $CL_R$, ml/min | 3.9 ± 1.2 | 1.4 ± 0.9** | 2.4 ± 0.7† | 2.8 ± 1.3† |
| $CL_{CR}$, ml/min | 1.7 ± 0.3 | 0.9 ± 0.5** | 1.4 ± 0.2† | 1.7 ± 0.4† |
| $CL_R - CL_{CR}$ | 2.3 ± 1.0 | 0.5 ± 0.5** | 1.0 ± 0.6* | 1.1 ± 1.0* |

Values are means ± SD; $n = 6$/group. AUC area under the curve; $T_{1/2\alpha}$, distribution-phase half-life; $X_{Bile}$, biliary excretion; $X_{Urine}$, urinary excretion; $CL_{Tot}$, total clearance; $Vd_{ss}$, steady-state volume of distribution; $CL_{bile}$, biliary clearance; $CL_R$, renal clearance; $CL_{CR}$, creatinine clearance; $CL_{RS}$, tubular secretory clearance of azithromycin. *$P < 0.05$, **$P < 0.01$, significantly different from control values. †$P < 0.05$, ††$P < 0.01$, significantly different from LPS values.

a change in $NO_2$ levels. However, total NO in plasma was decreased by both agents. The only substrate for NOS is arginine, which is converted to NO and citrulline. As a result, lowered plasma levels of arginine and an enhanced concentration of citrulline may be found in sepsis, which also complies with our results (Fig. 5*F*). Arginine deficiency in such conditions may contribute to a number of negative effects, such as endothelial and T cell dysfunction (34). Importantly, the administration of both agents in our study restored the citrulline/arginine ratio in serum and attenuated the LPS-mediated induction of TNF-α and IL-6 mRNA in the kidneys (Table 6).

## DISCUSSION

The main finding of the present study is that nonselective immunosuppression by a corticosteroid or the selective IL-1 receptor blockade, by anakinra, may both alleviate endotoxemic renal impairment, especially by preserving glomerular filtration. On the other hand, the endotoxemic depression of renal tubular functions, including drug excretory capacity, was only partially influenced by the agents. While diverse effects were detected in LPS-downregulated basolateral transporters, the prevention of LPS-mediated upregulation was seen in the apical excretory Mrp2, which, however, had no effect on the excretion of its substrate, AZT. Impairment of tubular excretory functions of endobiotics, namely urea and bile acids, was favorably influenced by dexamethasone.

The positive modulation of some signs of AKI during endotoxemia, especially the restoration of GFR, has already been described for corticosteroids and is mainly ascribed to the downregulation of iNOS (25, 40, 47, 48). We have verified this mechanism for dexamethasone with the reduction of iNOS, NO plasma levels and the citrulline/arginine ratio, which is the indicator of arginine breakdown by NOS (31). Importantly, unlike in other forms of AKI, such as ischemic or cisplatin induced (1), only the IL-1 receptor blockade in our study showed that crucial functional parameters of LPS-imposed AKI were significantly related to this pathway, and could be improved by anakinra to a similar extent as to that with the use of dexamethasone. In fact, Berry et al. (4) recently suggested that IL-1β-mediated neutrophil recruitment may play a key role in the development of AKI, and even sole administration of IL-1β can produce a systemic and kidney response closely resembling an endotoxemia insult (42). In agreement, both compounds reduced renal IL-6 mRNA, confirming their anti-inflammatory influence, but predictably anakinra was unable to change TNF-α expression. On the other hand, contradictory results, with the absence of any beneficial effect of the IL-1β receptor blockade or IL-1RA during endotoxemia/sepsis, also

Table 6. *Gene expression of renal drug transporters and cytokines measured in kidneys*

| | Ctrl | LPS | DEX-LPS | ANA-LPS |
|---|---|---|---|---|
| *Abcc2* (Mrp2) | 100 ± 20 | 266 ± 298* | 137 ± 35† | 91 ± 28† |
| *Abcc4* (Mrp4) | 100 ± 8 | 180 ± 56* | 161 ± 57 | 156 ± 42 |
| *Abcb1a* (Mdr1a) | 100 ± 23 | 58 ± 23* | 63 ± 28** | 46 ± 13** |
| *Abcb1b* (Mdr1b) | 100 ± 64 | 184 ± 109* | 58 ± 26† | 83 ± 35† |
| *Abcb11* (Bsep) | 100 ± 71 | 50 ± 20 | 502 ± 71***††† | 112 ± 52 |
| *Slc10a2* (Asbt) | 100 ± 27 | 161 ± 55* | 43 ± 30**††† | 172 ± 90 |
| *Slc22a6* (Oat1) | 100 ± 17 | 41 ± 19*** | 43 ± 7*** | 77 ± 29*†† |
| *Slc22a7* (Oat2) | 100 ± 52 | 18 ± 17** | 45 ± 32** | 36 ± 21** |
| *Slc22a8* (Oat3) | 100 ± 23 | 39 ± 10** | 43 ± 16** | 69 ± 38* |
| *Slc22a1* (Oct1) | 100 ± 27 | 77 ± 33 | 45 ± 16* | 88 ± 33 |
| *Slc22a2* (Oct2) | 100 ± 30 | 51 ± 19* | 116 ± 49††† | 69 ± 16 |
| *Slc22a3* (Oct3) | 100 ± 27 | 46 ± 25* | 70 ± 25 | 81 ± 38 |
| *Slc47a1* (Mate1) | 100 ± 45 | 40 ± 17** | 44 ± 23** | 43 ± 13** |
| *Slc47a2* (Mate2) | 100 ± 36 | 82 ± 18 | 97 ± 34 | 96 ± 13 |
| *Il-6* (IL-6) | 100 ± 40 | 15,141 ± 7,085*** | 1,188 ± 770†††  | 8,335 ± 2,913**†† |
| *Tnf* (Tnf-α) | 100 ± 69 | 673 ± 210*** | 345 ± 243*†† | 694 ± 125*** |

Values are means ± SD presented as % of control values (measured in control saline-treated animals); $n = 6$/group. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, significantly different from control values. †$P < 0.05$, ††$P < 0.01$, †††$P < 0.001$, significantly different from LPS values.

Table 7. *Changes in mRNA expression of selected genes in rats after 4-h clearance study with azithromycin (14 h after administration of LPS)*

| | Ctrl | LPS | DEX-LPS | ANA-LPS |
|---|---|---|---|---|
| *Slc22a6* (Oat1) | 100 ± 33 | 43 ± 27** | 18 ± 6*** | 63 ± 24* |
| *Slc22a7 (Oat2)* | 100 ± 64 | 28 ± 20* | 84 ± 34 | 48 ± 26 |
| *Slc22a8 (Oat3)* | 100 ± 45 | 49 ± 31* | 9 ± 6***† | 79 ± 29 |
| *Slc10a2* (Asbt) | 100 ± 34 | 290 ± 106*‡ | 19 ± 14*††† | 116 ± 32††† |
| *Abcc2* (Mrp2) | 100 ± 22 | 124 ± 90* | 63 ± 27‡‡ | 78 ± 24 |

Values are means ± SD presented as % of control values (measured in control saline-treated animals; $n = 6$/group. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, significantly different from control values. $^{\dagger}P < 0.05$, $^{\dagger\dagger\dagger}P < 0.001$, significantly different from LPS values. $^{\ddagger}P < 0.05$, $^{\ddagger\ddagger}P < 0.01$, significantly different from corresponding values measured after 10 h from LPS administration.

exist (37, 52). Whether these discrepancies are a matter of appropriate dosage, or of pharmacological differences between IL-1RA and anakinra, requires further characterization. Knowing the short half-life of anakinra in rats, and based on a previous study on the prevention of liver impairment (16), we therefore selected a repeat dose administration after 5 h, which produced a reliable effect.

CO, another gaseous product significantly involved in cell signaling, could also be very important for the pathogenesis of AKI. Unlike NO, the role of CO released from hemoglobin by inducible HMOX1 is mainly considered nephroprotective (35). Tracz et al. (46) demonstrated that the LPS challenge in HMOX1$^{-/-}$ mice led to a greater activation of NF-κB in the liver and an elevation of serum cytokines and chemokines, while Vanova et al. (50) showed significant alleviation in LPS-induced hepatic damage by CO inhalation. On the other hand, Poole et al. (39) detected improved hemodynamic and renal hallmarks of endotoxemic AKI, after the administration of the HMOX1 inhibitor zinc protoporphyrin. The authors ascribed the effect to local NO release, which may occur in decreasing concentrations of CO. However, both drugs in our study prevented the LPS-mediated induction of CO production, with a parallel reduction of NO plasma levels. Upon consideration that negative regulation was described between NO and CO during sepsis (15), we suggest that changes in CO release in our study reflected the severity of inflammation during endotoxemia, rather than causal protective mechanisms.

Endotoxemia, as well as sepsis, has a critical negative impact on transported mediated drug elimination in all excretory organs (17). The new information provided by our study is the influence of both evaluated agents on renal drug-transporting proteins during endotoxemia. Previous studies described downregulation of Oat1, Oat3, or Oct1-3 basolateral transporters (21, 23), and inconsistent induction (Mdr1, Mrp2, or Bsep) (18–20), maintenance (Mdr1, and Mrp2) (5, 55), or even downregulation (Mdr1) (2) of apical tubular proteins in LPS animals. Importantly, in these studies such modifications were associated with corresponding changes in the kinetics of sub-

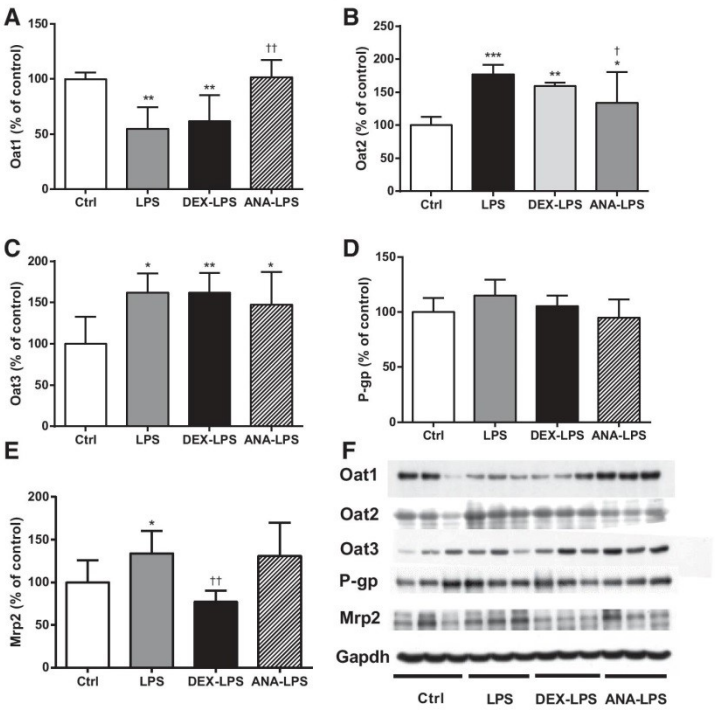


Fig. 3. Protein expression of renal drug transporters Oat1-3, Mrp2, and P-gp as determined by Western blotting in control (Ctrl), LPS-administered (LPS), and DEX- or ANA- pretreated LPS rats. Values are means ± SD ($n = 6$). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, compared with controls. $^{\dagger}P < 0.05$, $^{\dagger\dagger}P < 0.01$.

Table 8. *Liver gene expression of major bile acid and drug-transporting proteins*

| | Ctrl | LPS | DEX-LPS | ANA-LPS |
|---|---|---|---|---|
| *Slc10a1* (Ntcp) | 100 ± 15 | 4 ± 2*** | 22 ± 8***†† | 9 ± 4*** |
| *Abcb11* (Bsep) | 100 ± 32 | 20 ± 5*** | 62 ± 39*† | 22 ± 3*** |
| *Slco1a2* (Oatp1a2) | 100 ± 46 | 2 ± 1*** | 18 ± 16*** | 3 ± 3*** |
| *Abcc2* (Mrp2) | 100 ± 34 | 2 ± 1*** | 17 ± 18*** | 2 ± 1*** |
| *Abcc3* (Mrp3) | 100 ± 42 | 52 ± 28*** | 26 ± 10*** | 44 ± 12*** |
| *Abcc4* (Mrp4) | 100 ± 16 | 115 ± 20 | 153 ± 25**† | 170 ± 37***† |
| *Abcb1b* (Mdr1b) | 100 ± 72 | 1,017 ± 780** | 337 ± 265† | 1,409 ± 415***††† |

Values are means ± SD presented as % of control values (measured in control saline-treated animals); $n = 6$/group. $^{*}P < 0.05$, $^{**}P < 0.01$, significantly different from control values. $^{\dagger}P < 0.05$, $^{\dagger\dagger}P < 0.01$, $^{\dagger\dagger\dagger}P < 0.001$, significantly different from LPS values.

strates for these transporters. Our data from the LPS group is mainly consistent with these observations. The only discrepancy was in the up- instead of downregulation of the Oat3 transporter, and currently there is no explanation for this effect. Concerning renal tubular apical proteins, we support the transcriptional induction of multidrug transporters during endotoxemia (20), which was formerly ascribed to the inducing effect of cumulating anionic substrates, such as bile acids (18). However, a similar reduction of plasma bile acid by both agents, and differential changes in multidrug protein contents, suggest the existence of another mechanism. Indeed, dexamethasone is a well-known enzyme inducer, and through stimulation of the PXR receptor (38), it is also able to increase the expression of Mdr1 and Mrp2 in excretory organs, including the kidneys of healthy animals (32, 33). However, its administration in our study led to the downregulation of Mrp2, which points to a prevailing regulation of these transporters during endotoxemia by inflammatory mediators, which were effectively suppressed by the agent. In agreement, Heemskerk et al. (19) showed that the upregulation of Mdr1 is also NF-κB dependent, which is the transcription factor suppressed by corticosteroids (7). In contrast, anakinra changed Oat1 and Oat2 protein, which signifies the regulatory role of the IL-1 receptor in their posttranscriptional regulation.

The function of renal drug transporters was evaluated by the measurement of the clearance parameters of azithromycin, which is the substrate for Mdr1 and Mrp2 (45). The $CL_R/CL_{CR}$ ratio of 2.6 showed a marked secretion of the drug in the renal tubuli of control animals, and its significant decrease in LPS animals, despite the transcriptional upregulation of Mrp2 and

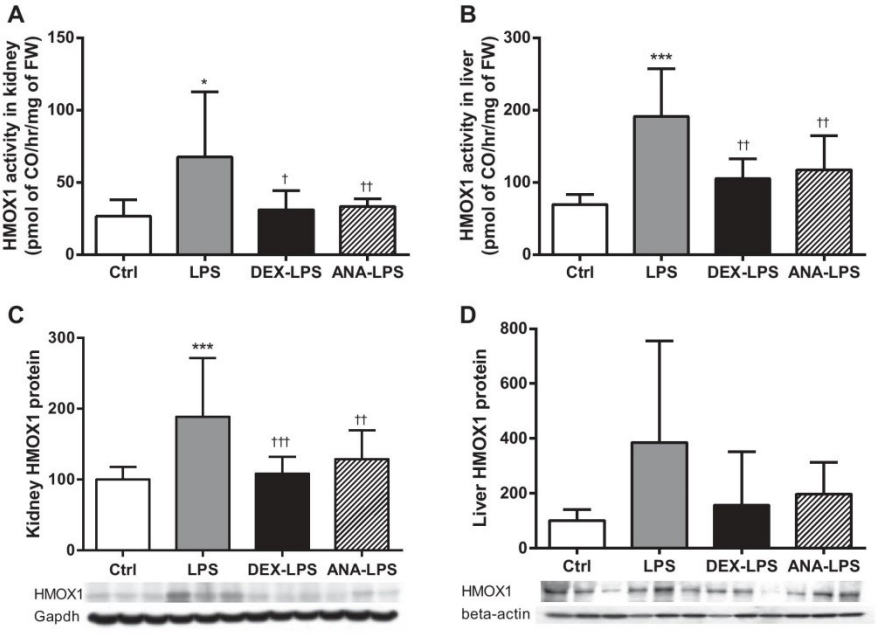


Fig. 4. Heme oxygenase-1 (HOMX1) activity (*A* and *B*) and protein expression (*C* and *D*) in the kidneys and liver of control (Ctrl), LPS-administered (LPS), and DEX- or ANA- pretreated LPS rats. Values are means ± SD ($n = 6$). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, compared with controls. $^{\dagger}P < 0.05$, $^{\dagger\dagger}P < 0.01$, $^{\dagger\dagger\dagger}P < 0.001$, compared with LPS.

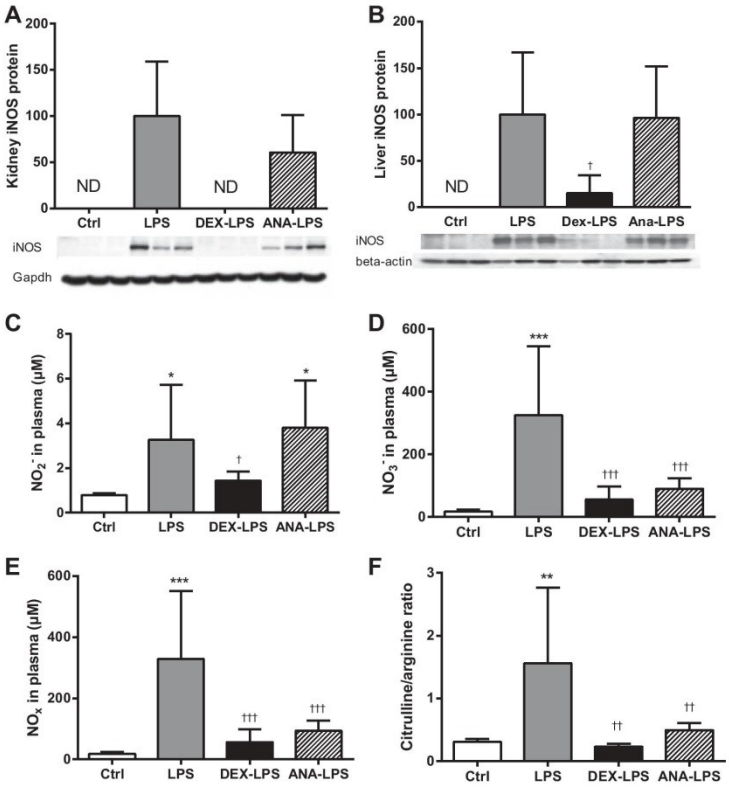


Fig. 5. Inducible nitric acid synthase (iNOS) expression in the kidneys (*A*) and liver (*B*), concentrations of $NO_2$ (*C*), $NO_3$ (*D*), and NOx (*E*) in plasma, and the plasma citrulline/arginine ratio (*F*) in control (Ctrl), LPS-administered (LPS) and DEX- or ANA-pretreated LPS rats. Values are means ± SD ($n = 6$). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, compared with controls. $^{\dagger}P < 0.05$, $^{\dagger\dagger}P < 0.01$, $^{\dagger\dagger\dagger}P < 0.001$, compared with LPS.

the maintenance of Mdr1 protein expressions, respectively. Such a discrepancy suggests that both transporters are not the only proteins responsible for azithromycin tubular secretion. Decreased basolateral uptake by Oat1 and increased apical reuptake by Oat2, the major renal transporters for organic anions, may indeed indicate the involvement of these molecules in AZT elimination (51). In support, both immunosuppressants were unable to increase tubular secretion of the antibiotic, because of diverse effects on the transporters. Although the relationship of AZT to transporters from the solute carrier family, SLC22, awaits further clarification, our data may have further implications. First, the comparison of mRNA and protein contents showed that during endotoxemia, transcriptional regulation prevails in renal Oat1, Mrp2, and Mdr1, while posttranscriptional modification is important for Oat2 and Oat3 and that these processes are dependent on IL-1 receptor stimulation. Second, corticosteroids or IL-1RA administration under such circumstances differentially modulate individual transporters and may modify the kinetics of their substrates, where drugs commonly administered in sepsis also belong, such as penicillins or cephalosporines (51).

Comparison of renal clearances of AZT and creatinine demonstrated that both therapies modified kinetics of AZT especially by changes in GFR. Although creatinine clearance is not considered as an optimal marker for GFR due its minor tubular secretion and reabsorption (49), reported values and degree of its reduction after LPS administration in our study are in agreement with former data reported with inulin (49, 54). Together with restoration of creatinine clearance in our endotoxemic animals administered with either immunosuppressant, despite uninfluenced expression of all transporters mediating creatinine secretion (Oct2/3, Mate1/2, and Oat2/3) (28), it suggests sufficient sensitivity of this marker for estimation of GFR in our settings. This assumption is further supported by corresponding changes in microalbuminuria, the marker of glomerular impairment. On the other hand, persistence of increased serum creatinine levels after administration of either immunosuppressant to endotoxemic animals despite normalization of creatinine clearances may reflect restoration of decreased creatinine production during sepsis (8), and/or incomplete attenuation of impaired kidney perfusion.

We, herein, also demonstrated that both compounds increased the biliary elimination of bile acids and reduced their levels, as well as other indicators (bilirubin, ALP) of cholestasis in plasma. In addition, we discovered that dexamethasone reduced the expression of the renal tubular bile acid uptake

transporter Asbt and increased the efflux, Bsep, with a consequent increase in bile acid renal clearance to control levels. Although the extent of renal bile acid elimination is much smaller than hepatic elimination, the increase in bile acid elimination after the administration of dexamethasone is interesting, because the drug has been recently described as a promotor of cholestasis, due to the inhibition of the transcriptional activity of the farsenoid-X receptor (30), which is the bile acid sensor. Transcriptional upregulation of the farsenoid-X receptor target gene Bsep in the kidneys and liver by dexamethasone and its absence after the administration of anakinra, signify the involvement of another, as yet unknown, regulatory pathway. This consideration is also supported by the lack of the anakinra effect on the other tubular excretory functions, whereas dexamethasone restored the fractional excretion of urea, the change attributable to the upregulation of urea transporters (41), and raised the fractional excretion of sodium, which is well known during corticosteroid therapy and reflects their stimulatory effect on sodium transportation in tubuli (43).

Although we are aware of the limitations of the tested animal model of sepsis for the generalization of obtained results, reported similarities allow us to conclude that our results support the use of the presented immunosuppressants for the prevention of AKI during sepsis and for the partial compensation of the deficit in renal drug elimination under these circumstances, especially with the restoration of GFR. However, marked variability in modulation of tubular secretion of AZT by both agents in our study suggests that significant augmentation of active tubular secretion of drugs can be expected in some individuals in sepsis administered with immunosuppressant, what may contribute to high interindividual variability in the elimination of coadministered agents. In addition, we have demonstrated the involvement of the IL-1 pathway in the regulation of drug excretory mechanisms during endotoxemic AKI, and also presented the complex influence of corticosteroids, the agents currently used in this indication. Our data also point towards the involvement of transporters, other than multidrug-resistance transporters, in the urinary elimination of AZT.

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#### DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

#### AUTHOR CONTRIBUTIONS

Author contributions: Z.K., E.D., J. Cermanova, M. Hroch, T.L., L.M., J. Chladek, and Z.H. performed experiments; Z.K., E.D., J. Cermanova, M. Hroch, T.L., L.M., F.S., L.V., J.M., J. Chladek, Z.H., M. Holecek, and S.M. analyzed data; Z.K. and S.M. prepared figures; Z.K. approved final version of manuscript; E.D. and S.M. provided conception and design of research; E.D., T.L., F.S., L.V., M. Holecek, and S.M. interpreted results of experiments; J. Cermanova and S.M. drafted manuscript; L.V., J.M., and M. Holecek edited and revised manuscript.

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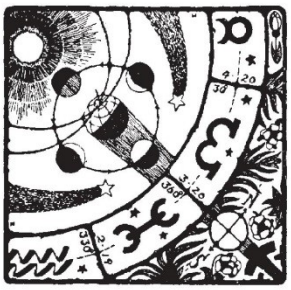

## 7.2. Deferoxamine but not dexrazoxane alleviates liver injury induced by endotoxemia in rats.

Cermanova J, Kadova Z, Dolezelova E, Zagorova M, Safka V, Hroch M, Laho T, Holeckova M, Mokry J, Kovarikova P, Bures J, Sterba M, Micuda S.

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Intracelulární železo kumulující se v játrech během endotoxémie vede ke zvýšené tvorbě volných radikálů s následným poškozením tkáně. Jedním z nadějných přístupů pro prevenci tohoto poškození jater se proto jeví snížení dostupnosti katalyticky aktivního volného železa ve tkáni použitím chelátorů.

Extracelulární chelátor železa deferoxamin (DFO) snižuje oxidační poškození životně důležitých orgánů, jako jsou plíce a ledviny a zvyšuje procento přežití u septických pacientů. Mezi další funkce patří redukce funkcí monocyt-makrofágového systému, která je dále podpořena sníženou produkcí prozánětlivých cytokinů jako TNF-alfa. Dalším chelátorem v této studii byl dexrazoxan (DEX), který dobře proniká do intracelulárního prostředí, kde je metabolizován na sloučeninu velmi blízkou EDTA. Dexrazoxan je schválen pro klinické využití jako kardioprotektivum v antracyklinové kardiotoxicitě, kdy působí jako chelátor volného nebo slabě vázaného léčiva. Vzhledem k tomu, že DEX se metabolizuje na ADR-925 v játrech, je ideálním kandidátem k prevenci endotoxémií navozeného poškození jater. Cílem této studie bylo porovnat účinek dvou chelátorů železa během modelového septického stavu indukovaného endotoxinem a s tím spojeného poškození jater.

Lipopolysacharid snížil plazmatické koncentrace železa spolu se zvýšenou produkcí hepcidinu a sníženou expresí ferroportinu v játrech. Přesto, že oba chelátory byly schopny snížit obsah železa v játrech s odpovídajícími změnami v expresi hepcidinu a ferroportinu, pouze DFO ukázal protektivní účinek proti poškození jater. Bohužel DEX neprokázal ani jeden ze znaků protekce. Naopak ještě zhoršil poměr GSH/GSSG, což je hlavní indikátor oxidativního stresu. Tato studie tedy prokázala výrazné rozdíly v účinku dvou klinicky používaných chelátorů na modulaci endotoxinem navozené jaterní patologie.

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# DEFEROXAMINE BUT NOT DEXRAZOXANE ALLEVIATES LIVER INJURY INDUCED BY ENDOTOXEMIA IN RATS

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ABSTRACT—The purpose of the present study was to compare the activity of two different clinically available iron chelators on the development of acute liver injury after administration of the bacterial endotoxin (lipopolysaccharide [LPS]) in rats. Lipopolysaccharide was administered either alone or after pretreatment with dexrazoxane (DEX) or deferoxamine (DFO). Control groups received only saline or its combination with either chelator. After 8 h, untreated LPS rats developed liver injury, with signs of inflammation and oxidative stress. Lipopolysaccharide reduced plasma iron concentrations in association with increased production of hepcidin and the reduced liver expression of ferroportin. Administration of chelating agents to LPS animals showed distinct effects. Although both drugs were able to reduce liver iron content, together with corresponding changes in hepcidin and ferroportin expressions, only DFO showed a protective effect against liver injury despite relatively small liver concentrations. In sharp contrast, DEX failed to improve any hallmark of liver injury and even worsened the GSH/GSSG ratio, the indicator of oxidative stress in the tissue. High-performance liquid chromatography–mass spectrometry analysis showed marked liver accumulation of iron-chelating metabolite of DEX (ADR-925), whereas the parent compound was undetectable. Further downregulation of transporters involved in bile formation was observed after DFO in the LPS group as well as in healthy animals. Neither chelator imposed significant liver injury in healthy animals. In conclusion, we demonstrated marked differences in the modulation of endotoxemic liver impairment between two iron chelators, implicating that particular qualities of chelating agents may be of crucial importance.

KEYWORDS—Deferoxamine, dexrazoxane, endotoxin, iron, liver impairment

## INTRODUCTION

The liver is one of the central organs for the onset, modulation, and termination of systemic toxicity produced by gram-negative bacteria during the sepsis (1). The molecules, which significantly contribute to the impairment imposed by these bacteria, are lipopolysaccharide (LPS) endotoxins, the major components of their outer membrane. In the liver, LPS binds to Toll-like receptors (TLRs) at the Kupffer cells and induces the production of nitric oxide (NO) and proinflammatory cytokines such as tumor necrosis factor-α (TNF-α), interkeukin-1β (IL-1β), and IL-6. These molecules, together with the direct effect of cumulating leukocytes and LPS (2), subsequently induce a stress response of hepatocytes characterized by generation of reactive oxygen species and corresponding protein and lipid membrane impairment (3). Hepatocellular damage is further worsened by downregulation of transporters responsible for bile acid–dependent, as well as –independent, bile production with consequent intrahepatic cholestasis (4).

Recent advances strongly suggest that the liver may significantly modulate the pathophysiology of sepsis/endotoxemia among others by targeting iron metabolism. Bacteria require sufficient plasma concentration of free iron for proper proliferation. One of the spontaneous defense mechanisms against bacterial growth is, therefore, a reduction of iron plasma levels, which is regularly seen during inflammatory situations (2). This effect is mediated by increased liver production of the main iron-regulating peptide—hepcidin (5). Unsurprisingly, hepcidin was first identified as an antimicrobial peptide in plasma and urine (6) but, consequently, it was described as the main suppressor of iron absorption in the duodenum and inducer of intracellular iron sequestration in hepatocytes and macrophages. High hepcidin production, therefore, leads to a reduction of the iron in the serum. At the molecular level, hepcidin binds to ferroportin, the only exporter of iron from the cells, with subsequent internalization of the transporter and its degradation. Although the principal pathway upregulating hepcidin production is the iron-sensitive BMP-SMAD (bone morphogenetic protein–son of mothers against decapentaplegic) cascade, its synthesis is also induced during the inflammation by cytokine IL-6, which in turn activates pSTAT3-controlled transcription (7). Thus, the intracellular labile iron pool consequently increases during endotoxemia, which may contribute

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to the generation of free radicals via Fenton reaction and accentuating the liver injury (8). In such situations, one of the reasonable approaches for the reduction of tissue injury might be the reduction of the availability of catalytically active free iron in the tissue.

Indeed, administration of iron chelator deferoxamine (DFO) during sepsis or endotoxemia has been repeatedly reported to reduce oxidative damage in vital organs, such as the lungs or kidneys, and it may even lead to improved survival (9–14). The association of this effect with the ability to reduce the function of the monocyte-macrophage system is suggested by the corresponding decrease in the production of key regulatory cytokines such as TNF-α (13, 15). However, the therapeutic potential of iron chelation is far from being fully understood and, particularly, sparse data are available regarding its ability to modulate endotoxemia-induced liver injury. Furthermore, data in the literature were obtained almost exclusively with DFO, which is still the gold standard for treatment of iron overload diseases in clinical practice, but its hydrophilicity makes intracellular access possible only by endocytosis and limits its intracellular distribution mainly to endosomes and lysosomes (16, 17), which may be a significant limitation in animals without severe iron overload. In fact, the only iron chelator, which has been found to provide convincing tissue-protective effects in animals and patients not suffering from marked iron overload, is a bisdioxipiperazine derivative, dexrazoxane (DEX). Dexrazoxane is traditionally believed to be a prodrug that easily penetrates into the intracellular compartment, where it is metabolized to the metal-chelating compound ADR-925, which is structurally very close to ethylenediaminetetraacetic acid (EDTA) (18). The drug is approved for clinical use as a cardioprotectant in anthracycline cardiotoxicity settings and also in accidental anthracycline extravasation. Its tissue-protective effects are attributed to the intracellular chelation of free or loosely bound iron, which should prevent reactive oxygen species formation, although alternative mechanisms have also been proposed (18). Because DEX is known to be metabolized to ADR-925 also in the liver, it may appear as an ideal candidate for the prevention of endotoxemia-induced liver injury. Unfortunately, no data on potential protective effects of this drug in these settings are available in the literature.

The aim of the present study was to compare the effect of DFO and DEX as two different iron-chelating agents on iron homeostasis, inflammation, and liver injury imposed in rats by endotoxin administration. The distribution of both agents into the liver was verified by high-performance liquid chromatography–mass spectrometry (HPLC-MS) method in healthy as well as endotoxemic animals.

## MATERIALS AND METHODS

### Animals

Male Wistar rats (SPF, Anlab, Prague, Czech Republic) with initial weight of 280 to 310 g were used throughout the study. The animals were housed under controlled environmental conditions (12-hour light-dark cycle; temperature, 22°C ± 1°C), with commercial food and water freely available. All experiments were performed in accordance with the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH publication, 1996) and under the supervision of the Ethical Committee of the Faculty of Medicine in Hradec Kralove.

### Experimental design

The animals were divided into three experimental groups and one control group (eight animals per group). Rats in the experimental groups were injected i.v. with endotoxin (LPS from *Salmonella typhimurium*, 4 mg/kg body weight, dissolved in saline). Although animals in the first group were injected with LPS alone, the other two LPS groups were pretreated with i.v. DEX (50 mg/kg, Cardioxane injection; Novartis) or DFO (50 mg/kg, Desferal injection; Novartis) 1 h before LPS administration; the control group was injected with saline. The animals were euthanized by exsanguination from the abdominal aorta 8 h after LPS/saline administration under pentobarbital anesthesia (50 mg/kg, i.p.), and plasma and organs were harvested for the evaluation. The 8-h insult was chosen on the basis of preliminary testing and also according to the previously reported hepatic damage imposed by LPS (8), as an optimal combination between continuous inflammatory response, onset of liver injury, and changes in iron and bile acid homeostasis.

### Histological examination

Liver tissue was fixed in 10% neutral-buffered formalin and embedded in paraffin. Sections of 5-μm thickness were affixed to slides, deparaffinized, and stained with hematoxylin and eosin to determine morphologic changes induced by LPS and chelators. Olympus BX-51 microscope equipped with a digital DP-71 camera was used for image documentation.

### Analytical procedures

The biochemical analyses of plasma were performed by routine measurements on Cobas Integra 800 (Roche Diagnostics, Mannheim, Germany), according to the manufacturer's instructions. The content of iron in the liver was determined using graphite furnace atomic absorption spectrometry (Unicam, Solaar 959, UK). Before analysis, samples were dried and, after the cooling period, they were weighted and digested by microwave digestion with nitric acid and hydrogen peroxide. Hematology was detected from EDTA-stabilized blood samples on a Sysmex XE-2100 analyzer (Sysmex, Kobe, Japan). Differential cell counts were estimated in whole-blood smears stained pan-optically by the Pappenheim technique (May-Grünwald and Giemsa-Romanovsky). Concentrations of reduced and oxidized glutathione in biological samples were determined by liquid chromatography with fluorimetric detection (19).

### HPLC-MS analysis of DEX, ADR-925, and DFO

The analyses were performed on an LC 20A Prominence (Shimadzu, Germany) chromatographic system coupled on line with LCQ Advantage Max mass spectrometer (Thermo Finnigan USA). The data were processed using Thermo Finnigan Xcalibur software version 2.0.

Concentrations of DEX and ADR-925 in biological materials were assessed using a previously developed HPLC-MS method with minor modifications (20). Briefly, a Synergi Polar-RP column (150 × 3 mm, 4 μm; Phenomenex USA) and a gradient profile of a mobile phase composed of 5% methanol in 2 mM ammonium formate and methanol were used. Electrospray ionization in positive mode and selected reaction monitoring were used for detection and quantification. Samples were treated by precipitation with methanol. The linearity of the method for determination of DEX and ADR-925 in plasma was proved within the range of 2 to 340 μM and 1 to 100 μM for DEX and ADR-925, respectively. Linearity of the method for determination of the compounds in the liver homogenate was tested within the range of 1.8 to 180 nmol/g and 1.6 to 165 nmol/g for DEX and ADR-925, respectively. Samples with a higher ADR-925 content were appropriately diluted with methanol before analysis. The lowest concentrations tested herein represent the lower limit of quantification.

Analysis of DFO was performed using a Zorbax SB-Aq column (150 × 3 mm, 3.5 μm; Agilent Technologies USA) and a mobile phase composed of 2 mM ammonium formate (component A) and acetonitrile (component B) in a gradient mode (0 – 8 min 20% B; 8 – 8.01 min 20% – 60% B; 8.01 – 11 min 60% B; 11 – 11.01 min 60% – 20% B; 11.01 – 15 min 20% B). A flow rate of 0.3 μL/min, a column temperature of 25°C, and an autosampler temperature of 5°C were used. Electrospray ionization in positive mode and selected reaction monitoring were used in these analyses. Because of the robustness and detector response, DFO was quantified in the form of its complex with iron using the two most abundant fragments of the ion $[Fe(DFO\text{-}2H)]^+$: $m/z$ 614 → $m/z$ 414 and $m/z$ 496. Both plasma samples (50 μL) and liver homogenates (100 mg) were treated by precipitation with methanol, where 100 and 500 μL of methanol were added to plasma and liver homogenates, respectively. Complete conversion of DFO to its complex was assured by addition of 1 μL of 10 mM $FeCl_3$ solution before the treatment. Linearity of the method was tested within the range of 0.5 to 50 μM and 1.0 to 15 nmol/g of DFO in plasma and liver homogenate, respectively. The concentration of 0.5 μM was set to be a limit of detection for analysis of DFO in plasma, and 1.0 nmol/g was a lower limit of quantification for determination of DFO in liver homogenates.

TABLE 1. **The effect of administration of dexrazoxane and deferoxamine on selected serum biochemical parameters in healthy and endotoxemic rats**

| Parameter | Ctrl | Ctrl-X | Ctrl-F | LPS | LPS-X | LPS-F |
|---|---|---|---|---|---|---|
| Bile acids, μM | 9.2 ± 1.8 | 7.2 ± 0.7 | 14 ± 2.3 | 23 ± 2.4** | 21 ± 3.6** | 25 ± 3.1** |
| Urea, mM | 7.1 ± 0.3 | 6.8 ± 0.5 | 5.5 ± 0.3 | 18 ± 1.6*** | 15 ± 1.4*** | 17 ± 2.3** |
| Uric acid, μM | 25 ± 3.1 | 21 ± 5.3 | 25 ± 6.7 | 50 ± 6.8** | 40 ± 5.6* | 47 ± 4.4* |
| Creatinine, μM | 25 ± 1.4 | 21 ± 1.6 | 22 ± 1.4 | 37 ± 4.4** | 33 ± 1.8 | 36 ± 1.6** |
| ALT, μkat/L | 0.9 ± 0.1 | 0.9 ± 0.1 | 0.9 ± 0.1 | 1.5 ± 0.1*** | 1.4 ± 0.1*** | 0.9 ± 0.1††† |
| AST, μkat/L | 0.45 ± 0.03 | 0.55 ± 0.04 | 0.41 ± 0.04 | 1.3 ± 0.1*** | 1.4 ± 0.1*** | 0.6 ± 0.1††† |
| Total protein, g/L | 60 ± 2.0 | 53 ± 0.7 | 53 ± 0.5 | 54 ± 1.4** | 54 ± 1.1** | 54 ± 0.6** |
| Albumin, g/L | 40 ± 1.3 | 36 ± 0.8* | 37 ± 0.8 | 36 ± 0.7* | 35 ± 0.8** | 36 ± 0.5* |
| Glucose, mM | 10 ± 0.3 | 11 ± 0.9 | 9.5 ± 0.4 | 6.9 ± 0.5*** | 7.3 ± 0.3*** | 6.2 ± 0.3*** |

Data are means ± SEM (n = 8 in each group); significantly different from the control value (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$). Significantly different from the LPS value (†††$P < 0.001$).
Ctrl indicates control group; LPS, endotoxin administration; X, group pretreated with 50 mg/kg of dexrazoxane; F, group pretreated with 50 mg/kg of deferoxamine.

### *Real-time quantitative RT-PCR*

Examination of the designated gene expression was performed by qRT-PCR, as described previously (21). RNA was isolated from the liver tissue samples using TRIzol reagent (Invitrogen USA) and converted into cDNA with a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, Calif). Then, 30 ng of cDNA was loaded into reactions performed in triplicates. The amplifications were run using TaqMan Fast Universal PCR Master Mix and predesigned Taq-Man Gene Expression Assay kits provided by Applied Biosystems (see Table 1S, Supplemental Digital Contents 1, at http://links.lww.com/SHK/A226). The time-temperature profile used in the "fast" mode was 95°C for 3 min; 40 times: 95°C for 7 s, 60°C for 25 s. Two reference genes were selected for normalization, *Gapdh* (4352338E; Applied Biosystems) and *Ywhaz* (rYwhaz_Q1; GENERI BIOTECH s.r.o., Hradec Kralove, Czech Republic). The expression data were normalized by the geometric mean of both housekeeping genes. Finally, the relative expression between the control and affected groups was determined by comparison of normalized data.

### *Western blot*

The animal livers (200 mg) were minced in an ice-cold buffer (1 mL) 25 mM TRIS.HCl (pH = 7.6), 0.1% [wt/wt] Triton-X), containing 0.5 μg/mL leupeptin and 50 μg/mL benzamidine and homogenized with MagNA Lyser (Roche Diagnostics GmbH, Germany) 2 × 30 s at 6,000 rpm. Supernatants were obtained after a 10,000*g* centrifugation at 4°C for 10 min. Plasma was used for the assessment of transferrin and hepcidin expression. The protein concentration was determined with the BCA assay (Pierce, Rockford, Ill), and samples were stored at −80°C. One hundred micrograms of proteins were separated on 10% polyacrylamide gels. After the proteins were transferred to a PVDF membrane (GE Healthcare, Prague, Czech Republic), it was blocked for 1 h with 5% nonfat dry milk in Tris-buffered saline containing 0.05% Tween 20. The membrane was then incubated with primary and secondary antibodies for 1 h at the concentrations specified in Table 2S, Supplemental Digital Contents 1, at http://links.lww.com/SHK/A226. After washing the membrane four times with Tris-buffered saline containing 0.05% Tween 20 buffer, the chemiluminescence process and quantification of immunoreactive bands on the exposed films were carried out. Equal loading of proteins onto the gel was confirmed by immunodetection of β-actin.

### *Statistical analysis*

Experiments were carried out in eight animals per group. All experimental data are expressed as mean ± SEM. Differences between experimental and control values were assessed by one-way analysis of variance, followed by Newman-Keuls *post hoc* test, or unpaired *t* test, using GraphPad Prism 5.0 software (San Diego, Calif). A value of $P < 0.05$ was considered statistically significant.

## RESULTS

### *Biochemical, hematological, and histological examination*

Eight hours after the administration of LPS, we detected significant increases in serum levels of bile acids and in activities of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) as indicators of developing cholestasis and hepatocellular damage, respectively (Table 1). Simultaneously raised serum urea, creatinine, and uric acid reflected impairment of kidney function, whereas decreased serum levels of glucose and proteins are hallmarks of catabolic status. Lipopolysaccharide also markedly depressed the white blood cell count as a result of a drop in lymphocytes. In contrast, endotoxemia produced an increase in neutrophil content (Table 2). Whereas administration of DEX did not modulate any of these changes in LPS animals,

TABLE 2. **The effect of administration of dexrazoxane and deferoxamine on selected hematological parameters in healthy and endotoxemic rats**

| Parameter | Ctrl | Ctrl-X | Ctrl-F | LPS | LPS-X | LPS-F |
|---|---|---|---|---|---|---|
| Erythrocytes, $10^{12}$/L | 7.7 ± 0.1 | 7.4 ± 0.2 | 7.5 ± 0.1 | 7.1 ± 0.07 | 7.2 ± 0.15 | 6.9 ± 0.11 |
| Hemoglobin, g/L | 148 ± 4 | 143 ± 1.1 | 144 ± 2.3 | 143 ± 3.04 | 141 ± 2.9 | 140 ± 1.7 |
| Hematocrit | 0.42 ± 0.01 | 0.4 ± 0.01 | 0.4 ± 0.01 | 0.41 ± 0.01 | 0.41 ± 0.01 | 0.41 ± 0.01 |
| WBC count, $10^9$/L | 5.2 ± 0.3 | 4.7 ± 0.3 | 5.4 ± 0.4 | 2.9 ± 0.26*** | 2.6 ± 0.27 *** | 3.1 ± 0.24*** |
| Neutrophils (diff. count) | 0.27 ± 0.03 | 0.26 ± 0.04 | 0.24 ± 0.03 | 0.45 ± 0.02*** | 0.41± 0.03*** | 0.29 ± 0.02***††† |
| Lymphocytes | 0.7 ± 0.03 | 0.7 ± 0.03 | 0.7 ± 0.04 | 0.42 ± 0.05*** | 0.34 ± 0.03*** | 0.36 ± 0.03*** |

Data are means ± SEM (n = 8 in each group); significantly different from the control value (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$). Significantly different from the LPS value (†††$P < 0.001$).
Ctrl indicates control group; LPS, endotoxin administration; X, group pretreated with 50 mg/kg of dexrazoxane; F, group pretreated with 50 mg/kg of deferoxamine.

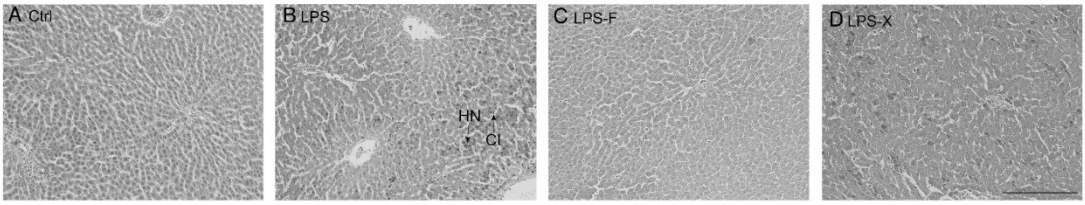


FIG. 1. **Histological analysis of hematoxylin-eosin–stained liver sections (scale bar, 500 μm)**. A, Control (Ctrl) group with normal liver morphology. Because the normal liver architecture was seen in other two control groups treated with either chelator, only a representative Ctrl picture is presented. B, LPS-treated rats exhibited hepatic injury with necrosis of hepatocytes (HN) and moderate cellular infiltration (CI). Whereas deferoxamine (LPS-F) attenuated LPS-mediated hepatic injury (C), dexrazoxane was without effect (D).

DFO premedication produced significant alleviation in ALT, AST activities, and neutrophil content. Administration of either chelator to the control animals did not considerably influence any of the measured parameters.

Histology of the rat liver sections in all control groups, including those receiving chelators, exhibited a normal integrated structure of hepatic lobule and intact hepatocytes without necrosis (Fig. 1). Challenge with LPS induced hepatic injury characterized by necrosis of hepatocytes with mild neutrophil infiltration. Chelators differentially modulated these alterations. Although use of DEX did not change the situation, DFO administration mitigated LPS-induced liver injury and reduced the incidence of hepatocyte necrosis and cell infiltration.

### *Iron biochemistry*

Plasma iron concentrations were markedly decreased in all LPS-treated animals, and pretreatment with either chelator had no significant impact on this event (Fig. 2A). Saturation of transferrin reflected the changes in plasma iron concentrations (Fig. 2B), and the administration of LPS led to its decrease in all treated groups, without modification after either drug. The total liver content of iron was unchanged by LPS but decreased significantly because of treatment by either chelator (Fig. 2C). Importantly, the administration of chelators to healthy rats resulted in a significant reduction of plasma iron concentrations after DFO and a marked reduction of liver iron content after either compound.

Levels of plasma transferrin were also reduced after LPS, despite its increased liver synthesis (Fig. 3A, B). Both chelators decreased transferrin protein levels in the liver but retained its plasma concentrations as compared with LPS alone. Hepcidin plasma levels, which were predictably increased in the untreated LPS group, were effectively reduced by both agents, with a more pronounced effect of DFO (Fig. 3C). Both chelators also prevented the LPS-induced expression of hepcidin and restored the ferroportin levels in the liver (Fig. 3D, E). Interestingly, the drop in liver hepcidin levels was deeper after DFO, which made hepcidin levels significantly lower in this group than in saline-treated controls. On the other hand, neither chelator modified these parameters in the control animals.

### *Plasma and liver concentrations of DEX and DFO*

To elucidate the liver distribution of chelators in our study, we used validated HPLC-MS methods for quantification of both parent drugs as well as ADR-925, an iron-chelating active metabolite of DEX (Table 3). As expected, DFO was detected in the liver tissue in a very low concentration, suggesting a relatively poor intracellular accumulation of the drug *in vivo*. Its concentrations in plasma were already below the detection limit, which is in line with the known short elimination half-life of the drug. Dexrazoxane also fell below the detection limit in plasma as well as in the liver, but its active metabolite was significantly concentrated in the tissue. Importantly, there was a significant difference in the liver distribution of both detected compounds between control and LPS groups. Lipopolysaccharide administration reduced liver concentrations of DFO but increased the accumulation of ADR-925.

### *Expression of genes involved in iron-liver homeostasis*

In line with the protein data previously presented, endotoxemia induced liver hepcidin *(Hamp)* mRNA (Table 4). This was accompanied by a reduction of ferroportin *(Slc40a1)* and transferrin receptor-1 *(Tfr1)* mRNAs, whereas gene expression of transferrin receptor-2 *(Tfr2)* and divalent metal transporter1 *(Slc11a2)* showed opposite regulation by endotoxemia. Noteworthy, although treatment with both chelators similarly

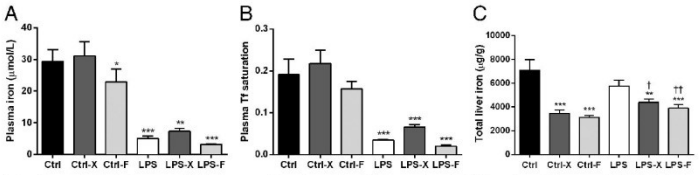


FIG. 2. **The effect of endotoxin and chelators on (A) plasma iron levels, (B) transferrin (Tf) saturation, and (C) total liver iron content calculated as grams of organ weight**. Ctrl indicates control group; LPS, LPS administration; X, animals pretreated with 50 mg/kg of dexrazoxane; F, animals pretreated with 50 mg/kg of deferoxamine. Values are expressed as mean ± SEM (n = 8 in each group); significantly different from the control group (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$); significantly different from the LPS group ($^{\dagger}P < 0.05$, $^{\dagger\dagger}P < 0.01$).

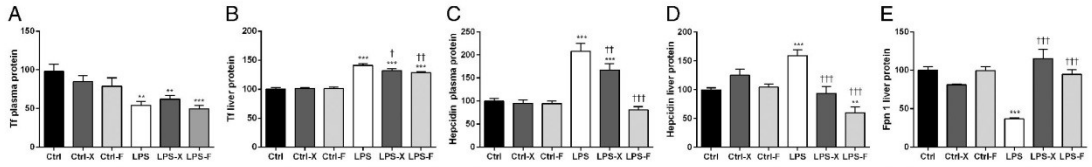


FIG. 3. **The effect of endotoxin and chelators on (A) hepcidin plasma protein, (B) hepcidin liver protein, (C) transferrin (Tf) plasma protein, (D) Tf liver protein, (E) ferroportin (Fpn1) liver protein**. Values are expressed as a percentage of the untreated control group. Ctrl indicates control group; LPS, LPS administration; X, animals pretreated with 50 mg/kg of dexrazoxane; F, animals pretreated with 50 mg/kg of deferoxamine. Values are expressed as mean ± SEM (n = 8 in each group); significantly different from the control group (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$); significantly different from the LPS group ($^{\dagger}P < 0.05$, $^{\dagger\dagger}P < 0.01$, $^{\dagger\dagger\dagger}P < 0.001$). Sample Western blots are presented in Figure, Supplemental Digital Contents 2, at http://links.lww.com/SHK/A227; Representative immunoblots for analyses presented by Figures 3 and 4. Ctrl indicates control group; LPS, LPS administration; X, animals pretreated with 50 mg/kg of dexrazoxane; F, animals pretreated with 50 mg/kg of deferoxamine.

reduced the gene expression of hepcidin, only DEX reduced the expression of *Tfr2*, *Tfr1*, and ferritin (*Ftl*) (Table 4). Moreover, administration of either chelator to control rats produced a downregulation of ferroportin, ferritin, Tfr1, and Tfr2 mRNA without influence on hepcidin mRNA.

### *Markers of inflammation and oxidative stress*

The modulation of liver inflammation was evaluated by detection of crucial regulatory molecules (Fig. 4). Untreated endotoxemic animals demonstrated a marked induction of expression of liver TNF-α and IL-6 genes, accompanied by increased protein content of both NOS isoforms. Administration of either chelator was effective in the attenuation of the previously described changes, with the exception of eNOS, which was significantly affected only by DFO. The oxidative stress response was evaluated by the changes in concentrations of GSH and GSSG (Fig. 5). Administration of LPS markedly reduced the liver concentration of reduced GSH and GSH/GSSG ratio, confirming induction of oxidative stress. Interestingly, the divergent reaction was detected after the administration of the chelators. Deferoxamine significantly decreased the endotoxemia-induced drop in both GSH levels and GSH/GSSG ratio, whereas DEX markedly raised GSSG concentration over LPS levels and further decreased the GSH/GSSG ratio. In untreated control animals, both drugs reduced liver concentrations of GSH and GSSG, which were more prominent for DEX, but the overall GSH/GSSG ratio was not modified.

### *Expression of genes involved in bile formation*

Untreated endotoxemic animals developed typical downregulation of the main transporters important for bile secretion, Ntcp, Bsep, Mrp2, Oatp1a4, as well as the genes encoding the rate-limiting enzyme for bile acid synthesis (Cyp7a1) and its regulator (Shp) (Table 4). Administration of DEX did not modify these changes, but DFO further downregulated Ntcp, Mrp2, and Oatp4. Interestingly, when compared with the untreated control group, a significant decrease in the expression of these genes was detected in healthy animals treated by either chelator.

## DISCUSSION

In the present study, we have compared the effect of two clinically available iron chelators, DFO and DEX, on the liver iron homeostatic mechanisms, inflammation, oxidative stress, and overall injury during endotoxemia. It should be noted that the chelators used in this study are considerably different in many aspects. Deferoxamine is a classic example of a highly selective iron chelator, which can directly interact with free or loosely bound iron to form redox-inactive chelates. However, its hydrophilic nature restricts intracellular distribution via passive diffusion. Indeed, the drug can enter the cells via endocytosis to get mainly to endosomes and lysosomes (16, 17), with variable access to the intracellular labile iron pool. On the other hand, DEX is a prodrug, which can get easily into the cells where it can be metabolized into an EDTA-like active metabolite ADR-925, but so far only limited data are available on its liver turnover (18). Therefore, we analyzed the concentrations of both agents in plasma and livers by sensitive HPLC-MS methods. Our data confirmed poor liver distribution of DFO. On the other hand, we detected high tissue concentrations of the hydrophilic metabolite ADR-925. This supports good penetration of the lipophilic parent compound into the hepatocytes and its rapid and effective metabolic conversion to the chelating metabolite. Furthermore, decreased liver distribution of DFO and increased deposition of ADR-925 in endotoxemic animals in comparison with the healthy animals signify different mechanisms used

TABLE 3. **Plasma and liver concentrations of deferoxamine and ADR-925 in rats**

| | Ctrl | | LPS | |
|---|---|---|---|---|
| | Plasma, μM | Liver, nmol/g | Plasma, μM | Liver, nmol/g |
| Deferoxamine | BDL | 4 ± 0.5 | BDL | 1.7 ± 0.1*** |
| ADR-925 | 1.3 ± 0.2 | 154 ± 6.6 | 3.4 ± 0.2*** | 316 ± 47** |

HPLC-MS measurement was performed 9 h after either chelator administration in healthy (Ctrl) or endotoxin-treated (LPS) animals. Dexrazoxane, the parent compound for ADR-925, was not detectable in the plasma or liver tissue of either group. Data are means ± SEM (n = 8 in each group); significantly different from respective control group (**$P < 0.01$, ***$P < 0.001$).
BDL indicates below detection limit.

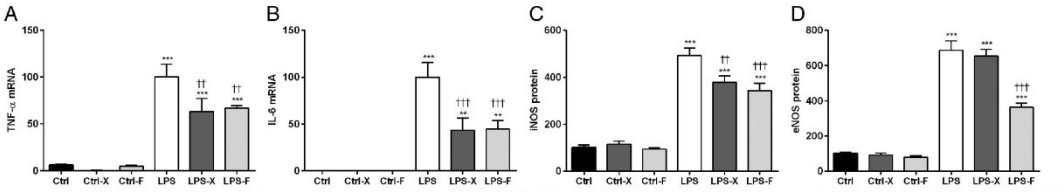


FIG. 4. **The effect of endotoxin and chelators on the liver expression of (A) TNF-α mRNA, (B) IL-6 mRNA, (C) inducible NO synthase (iNOS) protein, (D) endothelial nitric oxide synthase (eNOS) protein**. Ctrl indicates control group; LPS, LPS administration; X, animals pretreated with 50 mg/kg of dexrazoxane; F, animals pretreated with 50 mg/kg of deferoxamine. Values are expressed as mean ± SEM (n = 8 in each group); significantly different from the control group (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$); significantly different from the LPS group ($^{\dagger}P < 0.05$, $^{\dagger\dagger}P < 0.01$, $^{\dagger\dagger\dagger}P < 0.001$). Sample Western blots are presented in Figure, Supplemental Digital Contents 2, at http://links.lww.com/SHK/A227. Representative immunoblots for analyses presented by Figures 3 and Fig. 4. Ctrl indicates control group; LPS, LPS administration; X, animals pretreated with 50 mg/kg of dexrazoxane; F, animals pretreated with 50 mg/kg of deferoxamine.

in tissue kinetics of both agents. Involvement of bile excretory transporters that are typically impaired by LPS (4) may be expected in the case of accumulating ADR-925, whereas reduced uptake seems to dominate for DFO. These interesting data are novel and deserve further research.

The relationship between bacterial infection and iron turnover has been extensively studied (5). There is an agreement that the presence of bacteria, or their LPS, in circulation leads to the rapid iron sequestration to monocytes/macrophages. The rationale is to reduce the availability of iron for extracellular bacteria, with consequent inhibition of their growth (22). The effect is mediated by increased hepcidin production through an activated IL-6–GP130–STAT3 pathway in hepatocytes, LPS–TLR2/4–(BMP4/6)–MyD88–NFκB in macrophages (23–26), and perhaps also directly by activation of lipocalin 2/iron sequestrant systems in these immune cells (14). Importantly, iron, itself cumulating in macrophages, is able to stimulate production of proinflammatory cytokines, such as MCP-1 and IL-6, even without any other challenge, and such a response is further amplified in the presence of endotoxin (27–29). Hepcidin, in such a situation, may be the crucial feedback mechanism preventing excessive cytokine release after LPS because it directly limits IL-6 and TNF-α production in the liver and spleen macrophages by activation of Stat3-SOCS3 signal transduction. Its administration, or overexression, is associated with better survival of LPS-treated mice (30–32), whereas decreased hepcidin production may lead to increased frequency and severity of respiratory infections (33) or to proinflammatory status described in response to LPS during chronic iron deficiency with anemia (30). Hence, avid exchange of iron between plasma and monocyte-macrophage systems with rapid induction of hepcidin production may be the essential mechanism for the modulation of ongoing inflammation. The idea is also supported by the paradoxical finding that total liver iron content, that is, mainly in hepatocytes of our untreated LPS animals, was unchanged in comparison with that in healthy animals, a phenomenon which has also been reported by others (34, 35).

Series of works demonstrated that, during infection, acute short-term iron deprivation by either diet or chelation attenuated cytokine production and improved survival (13, 29, 35). A similar effect was seen in HFE-deficient mice, where the decreased intracellular free iron in macrophages blunts production of proinflammatory TNF-α and IL-6 in response to

TABLE 4. **Effect of LPS and chelators on the liver expression of genes involved in iron and bile acid homeostasis**

| Target gene | Ctrl | Ctrl-X | Ctrl-F | LPS | LPS-X | LPS-F |
|---|---|---|---|---|---|---|
| *Slc40a1* (Fpn1) | 100 ± 7.6 | 50 ± 3.4** | 60 ± 8.6** | 18 ± 2.7*** | 15 ± 2.2*** | 16 ± 2.3*** |
| *Ftl* (ferritin) | 100 ± 5.5 | 46 ± 9.4** | 46 ± 8.1** | 112 ± 8.2 | 79 ± 3.8* †† | 111 ± 12.1 |
| *Tfr1* (Tfr1) | 100 ± 6.1 | 67 ± 6.4*** | 54 ± 6.1*** | 54 ± 6.3*** | 24 ± 3.3*** †† | 44 ± 5.8*** |
| *Tfr2* (Tfr2) | 100 ± 8.4 | 63 ± 6.8** | 77 ± 8.4* | 127 ± 4.5* | 88 ± 5.6†† | 109 ± 16.2 |
| *Hamp* (hepcidin) | 100 ± 19 | 81 ± 9.2 | 117 ± 13 | 345 ± 14.9*** | 229 ± 23*** †† | 265 ± 23*** † |
| *Slc11a2* (Dmt1) | 0.9 ± 0.2 | 0.8 ± 0.01 | 0.9 ± 0.1 | 99 ± 34** | 24 ± 14†† | 25 ± 12†† |
| *Slc10a1* (Ntcp) | 100 ± 3.6 | 62 ± 20.3 | 41 ± 6.4** | 29 ± 2.6*** | 22 ± 3.3*** | 17 ± 1.7*** † |
| *Abc11* (Bsep) | 100 ± 6.1 | 69 ± 4.2* | 76 ± 11* | 41 ± 6.1*** | 30 ± 4.8*** | 28 ± 4.0*** |
| *Abcc2* (Mrp2) | 100 ± 11.2 | 51 ± 6.8** | 70 ± 7.4* | 3.3 ± 0.5*** | 2.6 ± 0.4*** | 2.1 ± 0.1*** † |
| *Slco1a2* (Oatp1a4) | 100 ± 8.5 | 43 ± 10* | 63 ± 12 | 35 ± 4.9*** | 23 ± 5.5*** | 14 ± 2.3*** †† |
| *Cyp7a1* (Cyp7a1) | 100 ± 17 | 75 ± 7.5** | 64 ± 5.9* | 3.7 ± 0.4*** | 4.9 ± 0.9*** | 7.4 ± 2.1*** |
| *NrOb2* (Shp) | 100 ± 14 | 31 ± 10* | 58 ± 11 | 9.9 ± 3.8*** | 2.2 ± 0.3*** | 5.3 ± 1.2*** |

Results expressed as percentages of the control group; exception was Dmt1 where LPS group was taken as 100% because of a low basal expression. Values are expressed as mean ± SEM (n = 8 in each group); significantly different from the control group (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$) or significantly different from the LPS group ($^{\dagger}P < 0.05$, $^{\dagger\dagger}P < 0.01$).
Ctrl indicates control group; LPS, endotoxin administration; X, group pretreated with 50 mg/kg of dexrazoxane; F, group pretreated with 50 mg/kg of deferoxamine.

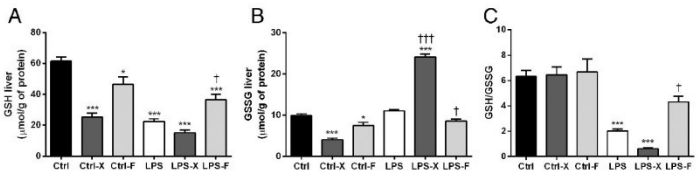


FIG. 5. **The effect of endotoxin and chelators on (A) liver concentrations of GSH, (B) liver concentrations of GSSG, and (C) liver GSH/GSSG ratio**. Ctrl indicates control group; LPS, LPS administration; X, animals pretreated with 50 mg/kg of dexrazoxane; F, animals pretreated with 50 mg/kg of deferoxamine. Values are expressed as mean ± SEM (n = 8 in each group); significantly different from the control group (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$); significantly different from the LPS group ($^{\dagger}P < 0.05$, $^{\dagger\dagger}P < 0.01$, $^{\dagger\dagger\dagger}P < 0.001$).

LPS. An investigation of the underlying mechanism revealed that low intracellular iron selectively impairs signaling via the TLR4/TRAM/TRIF pathway, decreasing the NF-κB late activation (36). Consistently, iron chelation by DFO has been shown to decrease NF-κB activation by LPS and prevents target cytokine production (35). The involvement of this mechanism is also apparent from our results. Both chelators induced similar decreases in the liver concentration of iron, in association with reduced expression of key NF-κB targets, TNF-α, IL-6, and iNOS. Similar efficacy of DEX and DFO suggests that both chelators had similar access to the key intracellular labile iron pool, which may be rather surprising considering the difference in the measured concentrations in the liver tissue (Table 3). However, although DFO cannot get into the target cells readily by passive diffusion, it can reach the intracellular compartment via endocytosis, which may be particularly important in Kupffer cells and macrophages playing a central role in inflammation.

An important finding was the discrepancy between both compounds in glutathione liver contents. Availability of the compound in the reduced form is essential for inactivation of reactive toxic species, including products of oxidative stress. The decreased ratio between reduced and oxidized forms in our untreated LPS group indicated increased oxidative stress, which corresponded with the ALT, AST, and histological status. Administration of DFO showed a positive influence on both parameters, in agreement with the effect on inflammatory markers. The described direct free radical scavenging capability of DFO (37) may contribute to this effect. In contrast, DEX worsened the oxidative stress and, finally, did not lead to biochemical or histological improvement. We suppose that such an untoward effect originates from the unique properties of the active metabolite of DEX, ADR-925. Similarly as EDTA, this compound interacts with free iron to form a complex $[Fe(ADR\text{-}925)\ H_2O]^+$, which contains a labile water molecule (18). As a result, the complex is not redox stable and, in the case of EDTA, it can be more or less liable to redox cycling depending on environmental conditions. This particular quality of ADR-925 may explain a lack of hepatoprotective potential of the drug despite the direct free radical scavenging ability of DEX (38). In agreement, analysis of tissue concentrations showed absence of DEX in the liver and accumulation of its active metabolite, which excludes the possibility of impaired drug distribution or metabolic conversion to the iron-chelating metabolite by the LPS treatment.

Reduction of liver iron content after administration of both drugs in control as well as endotoxemic animals complied with their chelating activity, but the effect was also followed by an increase in the protein content of the main iron-exporting molecule ferroportin in LPS-treated animals. Such upregulation may be related to the reduced internalization and degradation of the ferroportin molecule caused by reduced hepcidin production in the liver in response to reduced IL-6 (7). Persistent transcriptional downregulation of the *Fpn* gene could be ascribed to increased production of cytokines such as TNF-α (23). Interestingly, both chelators reduced the gene expression of ferritin, ferroportin, and transferrin receptors in control animals and DEX even in LPS animals. Because Tfr1 is inversely regulated by iron, the content of which was reduced by both chelators in the control as well as LPS animals (39), and neither chelator influenced hepcidin synthesis in the control animals, we anticipated that such uniform downregulation may occur through the direct effect of chelators. Nevertheless, this observation requires further attention.

Some studies have suggested nephroprotective mechanisms of iron chelation on kidney injury during endotoxemia/sepsis (9, 40). However, our biochemical data did not show any alleviation of the increased plasma concentration of creatinine, uric acid, or urea. Moreover, we have also demonstrated for the first time that the expression of several bile acids and drug transporters important within the process of bile formation were reduced by both chelators in the control animals and by DFO also in the LPS-treated animals. It indicates that iron may be involved in the regulation of these transporters as we have also recently seen in iron overloaded animals (unpublished data). Because endotoxemia is a well-known cholestatic condition developed because of downregulation of such transporters (4), as verified also in our study, this effect of chelators may, in this context, mean a potential for further worsening of the situation and is a risk factor also in healthy organisms. Consequently, reduced bile secretion may also be the reason why alleviation of other parameters of endotoxemic liver impairments after DFO was not associated with an improvement in plasma–bile acid concentrations. However, we are not aware of the existence of data examining the relationship of iron and regulation of the expression of these transporters, and further studies are required to elucidate these potentially harmful aspects of chelators.

In conclusion, the present study demonstrates the distinction in the ability of two iron chelators, DFO and DEX, to reduce liver injury during endotoxemia. Although both agents have comparable positive effects on the LPS-induced liver inflammation and hepcidin expression, their different abilities to protect the liver from oxidative stress and injury are noteworthy. Dexrazoxane not only left the endotoxin-mediated liver impairment unchanged but even worsened the oxidative stress in the liver. In

contrast, DFO rendered significant protective effects, and this was likely codetermined by its ability to reduce oxidative stress in the liver. However, the absence of any positive effect on kidney biochemistry and downregulation of some genes required for bile production in endotoxemic as well as in healthy animals may indicate novel untoward mechanisms of iron chelation during endotoxemia.

## ACKNOWLEDGMENTS

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## 7.3. Boldine enhances bile production in rats via osmotic and farnesoid X receptor dependent mechanisms.

Cermanova J, Kadova Z, Zagorova M, Hroch M, Tomsik P, Nachtigal P, Kudlackova Z, Pavek P, Dubecka M, Ceckova M, Staud F, Laho T, Micuda S.

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Boldin je jedním z hlavních alkaloidů izolovaných z listů a kůry boldovníku čilského (*Peumus boldus* Molina). Jeho účinky byly studovány na odpovídajících *in vivo* a *in vitro* modelech a výsledky potvrdily jeho silnou antioxidační aktivitu, která umožňuje prevenci před řadou onemocnění souvisejících s oxidačním stresem, jako jsou hepatotoxicita, zánětlivá onemocnění GITu, proliferace nádorových buněk a ateroskleróza. Jednou z hlavních vlastností připisovaných boldinu je jeho stimulační aktivita na tvorbu žluče, nicméně mechanismus tohoto účinku nebyl nikdy objasněn.

Cílem této studie bylo objasnit podstatu choleretického účinku boldinu u zdravých potkanů, ale také u potkanů s cholestázou, která byla navozena aplikací ethinylestradiolu. Tvorba žluči byla hodnocena bezprostředně po nitrožilní aplikaci látky i po 28-denní perorální premedikaci.

Naše studie ukázala, že boldin vykazuje výrazný akutní cholerecký účinek, který se objeví bezprostředně během jeho podání v závislosti na koncentraci dosažené ve žluči – prahová koncentrace pro dosažení efektu byla 10 μM. Mírný cholerecký efekt boldinu byl však patrný i po 28-denní premedikaci a tento účinek byl nezávislý na jeho přítomnosti v organismu. Mechanismus, který je za tímto dlouhodobým účinkem boldinu, je indukce Bsep zprostředkovaná přes transkripční faktor FXR (Farnesoid X receptor). Tato studie poprvé demonstrovala mechanismy a okolnosti cholerecického účinku boldinu, látky, která je volně dostupná v obchodní síti jako potravinový doplněk.

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# Boldine enhances bile production in rats *via* osmotic and Farnesoid X receptor dependent mechanisms

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ABSTRACT

Boldine, the major alkaloid from the Chilean Boldo tree, is used in traditional medicine to support bile production, but evidence to support this function is controversial. We analyzed the choleretic potential of boldine, including its molecular background. The acute- and long-term effects of boldine were evaluated in rats either during intravenous infusion or after 28-day oral treatment. Infusion of boldine instantly increased the bile flow 1.4-fold in healthy rats as well as in animals with Mrp2 deficiency or ethinylestradiol induced cholestasis. This effect was not associated with a corresponding increase in bile acid or glutathione biliary excretion, indicating that the effect is not related to stimulation of either bile acid dependent or independent mechanisms of bile formation and points to the osmotic activity of boldine itself. We subsequently analyzed bile production under conditions of changing biliary excretion of boldine after bolus intravenous administration and found strong correlations between both parameters. HPLC analysis showed that bile concentrations of boldine above 10 μM were required for induction of choleresis. Importantly, long-term pretreatment, when the bile collection study was performed 24-h after the last administration of boldine, also accelerated bile formation despite undetectable levels of the compound in bile. The effect paralleled upregulation of the Bsep transporter and increased biliary clearance of its substrates, bile acids. We consequently confirmed the ability of boldine to stimulate the Bsep transcriptional regulator, FXR receptor. In conclusion, our study clarified the mechanisms and circumstances surrounding the choleretic activity of boldine.



## Introduction

Bile formation (BF) is an essential function of the liver providing an important route for the excretion of many endo- and xenobiotics and depends on the transporter-mediated secretion of osmotically active compounds by hepatocytes and cholangiocytes. These compounds attract water to form bile in relation to their actual secretion. Two principal pathways are involved: bile acid-dependent bile flow (BADF), and bile acid-independent bile blow (BAIF) (Esteller, 2008). Regarding BADF, bile production is achieved by bile acids, the major endogenous osmotic bile constituents. Moreover, bile acids (BA) demonstrate strong indirect mechanisms, such as activation of the FXR (Farnesoid X receptor) nuclear receptor or post-transcriptional modulation with consequent change in expression, localization and finally function of bile acid transporters and enzymes responsible for bile acid synthesis and metabolism (Dombrowski et al., 2006; Boyer, 2013). Key to BAIF is the function of Mrp2 transporter (multidrug resistance-associated protein 2) which mediates biliary secretion of osmotically active glutathione (GSH). This tripeptide is the main mechanism of antioxidant defense protecting the hepatocytes against injury imposed by reactive oxygen species and electrophiles (Ribas et al., 2014). However, biliary secretion of GSH together with its oxidized form (GSSG) is also the major determinant of BAIF (Ballatori and Truong, 1992). As a consequence, modulation of mechanisms responsible for BADF or BAIF may in turn change the rate of elimination of their endogenous as well as exogenous substrates. Therefore characterization of influence of the agents administered to organism as drugs or food ingredients on these mechanisms may prevent serious interactions.

Choleretics are agents that stimulate bile production, and work through activation of BADF and/or BAIF, or may also possess direct

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osmotic activity when concentrated in bile (Ito et al., 2004; Shoda et al., 2004; Ruiz et al., 2005). In this respect, spironolactone represents a prototype substance inducing bile production indirectly by transcriptional up-regulation of Mrp2, with a consequent increase in biliary glutathione secretion (Ruiz et al., 2005, 2007). Although stimulation of pregnane X receptor (PXR) is the suspected cause for this effect (Ruiz et al., 2009), a direct link is missing. Benzylpenicillin, on the other hand, induces choleresis immediately after its administration through its own concentrative biliary excretion coupled with increased insertion of Mrp2 to the canalicular membrane. This is followed by enhanced biliary output of osmotically active glutathione (Ito et al., 2004; Fukami et al., 2011). Similar mechanisms have been identified in drugs such as valproate (Wright and Dickinson, 2004), nifedipine, or sodium nitroprusside (Yang and Hill, 2001) and for herbal choleretic remedies, especially cynarin (Speroni et al., 2003), curcumin (Deters et al., 1999), liquiritigenin (Kim et al., 2009) or genipin (Shoda et al., 2004).

Boldine is the major alkaloid from the leaf and bark of the Chilean Boldo tree (*Peumus boldus* Molina, Monimiaceae). It is responsible for the majority of the described health promoting activities of boldo extract, which was traditionally used to treat a wide variety of diseases and symptoms including headache, rheumatism, dyspepsia, urinary tract infections and sleep disturbances (O'Brien et al., 2006). The effects were studied using *in vitro* and *in vivo* models and the results indeed revealed the strong antioxidant activity of boldine, which enables prevention of various oxidative stress-related outcomes such as hepatotoxicity (Lanhers et al., 1991; Fernandez et al., 2009), inflammation (Backhouse et al., 1994), tumor cell proliferation, and atherosclerosis (Santanam et al., 2004). Interestingly, one of the major applications of the agent is its putative choleretic activity, which was recognized in earlier studies exploring boldo extracts. Initially, Delso-Jimeno (1956) described how the content of solids in bile increased without modifying the biliary volume following oral administration of boldine in dogs. Thereafter, a further series of works demonstrated that oral administration of boldo extract to mice or rats can also increase bile flow (Pirtkien et al., 1960; Borkowski et al., 1966; Levy-Appert-Collin and Levy, 1977). More recent studies have, on the contrary, failed to demonstrate choleretic activity after oral administration of 200–800 mg/kg of aqueous ethanolic boldo extract, or after intravenous administration of either ethanolic extract (4:1) or pure boldine itself (Lanhers et al., 1991). Together, discrepancies between these results indicate that better characterization of the possible mechanisms and conditions of putative choleresis after boldine are now required.

The aim of the present study was therefore to identify conditions required for the choleretic effect of boldine to occur in healthy rats as well as in animals with impaired BADF and/or BAIF mechanisms either induced by ethinylestradiol (EE) administration (Geier et al., 2007) or by congenital deficit of Mrp2 transporter (Johnson et al., 2006). To investigate the mechanisms responsible for effect, we analyzed bile acid dependent and independent pathways of bile formation either immediately after the compound bolus or continuous i.v. administration (to explain acute effects) or after its 28-day oral pretreatment (to describe the long-term effect).

## Methods

*Chemicals.* Boldine, ethinylestradiol (EE), chenodeoxycholic acid (CDCA), MK-571, and a reduced form of glutathione (GSH) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Boldine was dissolved in distilled water (pH adjusted by HCl to 1.0) with consequent titration to pH 7.0 by NaOH. Ethinylestradiol was dissolved in propylene glycol. All other chemicals were of the highest purity available and supplied by Sigma-Aldrich (St. Louis, CA, USA) or BioRad (Bio-Rad Laboratories, Hercules, USA).

*Animals.* Female Wistar rats (220–250 g, Velaz, Konarovice, CR), Mrp2 deficient Lewis TR$^-$ (transport-deficient) rats or complementary Lewis rats (both strains were a kind gift from Prof. Ingrid Kloting, Institut für Pathophysiologie, Karlsburg, Germany) were used throughout the study. All experimental protocols were conducted in accordance with the National Ministry of Agriculture guidelines and were approved by the Animal Care and Use Committee of the Faculty of Medicine in Hradec Kralove.

The design of pretreatments, substance administration during bile flow assessment, approach to blood sampling, and its duration were different according to the type of evaluation — Table 1. Bile collection study was performed in all experimental groups 24-h after the last pretreatment. Rats were anesthetized with sodium pentobarbital (50 mg/kg, intraperitoneally), fixed in a supine position on a heated platform to maintain body temperature at 37 °C, and v. jugularis (substance administration), carotid artery (blood sampling), and bile duct (bile collection) were cannulated. After an initial 30-min control period, the study was started with the i.v. administration of saline or boldine. All animals received i.v. 2.5 ml/h of fluids to replace loses by sampling. Bile was always collected in pre-weighted tubes at 30-min intervals. At the end of the experiment, rats were sacrificed by exsanguination from carotid artery, and samples of plasma, bile and livers were snap frozen in liquid nitrogen and stored at −80 °C until analysis.

*Cell culture.* Canine MDCKII cell line transduced with human *ABCC2* gene (MDCKII-MRP2), which stably expresses human MRP2 transporter and the corresponding parent MCDKII cell line, were provided by Dr. Schinkel and Prof. Borst (NKI-AVL, Amsterdam, Netherlands). The cells were grown in complete Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS). HepG2 cell line was purchased from the European Collection of Cell Cultures (Salisbury, UK) and was used within 25 passages after delivery. For transient transfection gene reporter experiments, HepG2 cells were maintained in antibiotic-free DMEM supplemented with 10% fetal calf

**Table 1**
Organization of animal experiments. Six-to-seven animals per group were used, only Mrp2 negative Tr$^-$ Mrp2 deficient rats were of 5 individuals. Bile was collected in all animals at 30-min intervals.

| Study | Pretreatment | Organization of bile clearance evaluation | | |
|---|---|---|---|---|
| | | i.v. administration | Blood sample (min) | Duration of infusion/bile collection (min) |
| $^{W}$I.v. infusion | Saline or EE (5 mg s.c. for 5 days)[a] | Continuous infusion of saline (2.5 ml/h) or boldine (50 mg/kg/h — in saline 2.5 ml/h)[a] | 15, 45, 75, 120 | 120 |
| $^{L/TR-}$i.v. infusion | – | Continuous infusion of saline (2.5 ml/h) or boldine (50 mg/kg/h — in saline 2.5 ml/h)[a] | 15, 45, 75, 120 | 120 |
| $^{W}$Boldine i.v. bolus dose | – | Bolus dose of boldine (50 mg/kg) by slow injection over 5 min[a] followed by continuous infusion of saline (2.5 ml/h) | 4, 10, 30, 60, 120, 180 | 180 |
| $^{W}$28-day pretreatment | Boldine 50 mg/kg/day p.o.[a] | Saline i.v. infusion (2.5 ml/h) | 15, 45, 75, 120 | 120 |

$^{W}$ — Wistar rats, $^{L}$ — Lewis rats, $^{TR-}$ — Mrp2 negative TR$^-$ rats; EE — ethinylestradiol (applied once daily 5 mg/kg body weight subcutaneously for 5 days) administered rats.
[a] Corresponding control groups received appropriate amount of vehiculum — saline (for boldine) or propylene glycol (for EE).

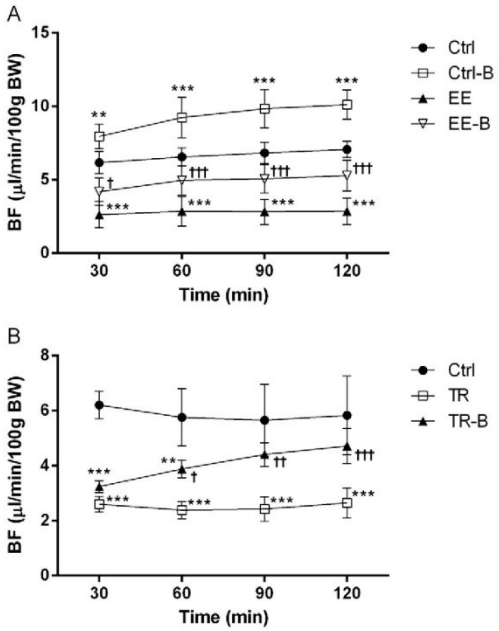


**Fig. 1.** Changes in bile production when boldine (B, 50 mg/kg) was administered by continuous intravenous infusion to A) healthy (Ctrl) or ethinylestradiol treated (EE – 5 mg/kg s.c. injection daily for 5 days) Wistar rats; B) Mrp2-negative TR$^-$ rats. Corresponding control rats received saline. Panel A data is from Wistar rats and panel B data is from Lewis rats (control and TR$^-$). Results are presented as means ± SD (A – n = 6–7 animals per group; B – TR$^-$ rats – n = 5 animals per group); significant difference from the control group (*P < 0.05, **P < 0.01, ***P < 0.001); significant difference from the TR$^-$ or EE group (†P < 0.05, ††P < 0.01).

serum, 1% sodium pyruvate, and 1% non-essential amino acids (all from Sigma-Aldrich).

*MRP2 efflux assay.* The efflux experiments were conducted 24 h after cell seeding ($2 \times 10^5$ cells per well on a 12-well plate). The medium was removed and MDCKII cells were washed twice with pre-warmed 1× phosphate buffered saline (PBS) at pH 7.4. Cells were then incubated for 10 min at 37 °C in 5% $CO_2$ in the presence of MK-571 (50 µM, a selective MRP inhibitor and Leukotriene D4 antagonist) or with six-point concentration scale of boldine diluted in Opti-MEM (0.1, 0.3, 1, 3, 10, 30 µM). Calcein AM, which is intracellularly converted to calcein, an MRP2 fluorescent substrate was added to reach the final concentration of 0.25 µM and allowed for 15 min accumulation phase. Then the efflux phase followed and cells were incubated for 1 h in the presence or absence of boldine (0.1, 0.3, 1, 3, 10, 30 µM). Efflux was stopped by cooling the plate, removal of medium and 2-times wash with ice-cold 1× PBS. The cells were detached with 10× trypsin–EDTA, resuspended in 1× PBS with 2% FBS and transferred to vials, which were placed on ice until analysis. The calcein fluorescence intensities of individual cells were analyzed using the C6 flow cytometer (Accuri, Ann Arbor, USA) on FL2 channel (ex 488 nm, em 585 nm).

*Gene reporter assay.* The HepG2 cells were co-transfected in 48-well plates with the pGL5-luc luciferase reporter construct (170 ng per well), pCMX-GAL4-FXR LBD expression plasmid containing Farnesoid X receptor (FXR) ligand binding domain (70 ng per well), and pRL-TK (30 ng per well) using Lipofectamine 2000 (Life Technologies). In other experiments, cells were co-transfected with the pFXRE-luc2P reporter construct (100 ng per well), pSG5-hFXR expression plasmid (100 ng per well), pSG5-hRXRα (70 ng/per well) and pRL-TK (30 ng per well) constructs using Lipofectamine 2000 (Life Technologies). Following 24 h of stabilization, HepG2 cells were exposed to various concentrations of boldine for 24 h (1, 5, 10, or 20 µM), or with chenodeoxycholic acid (CDCA; 20 µM, one of the main bile acids produced by the liver). DMSO (0.1% v/v) was used as a vehicle in all experiments. Cells were then lysed and analyzed for firefly luciferase activity normalized to enzyme activity of the Renilla luciferase (Dual-Luciferase Reporter Assay, Promega, Madison, WI). Data are presented as means with S.D. from three independent experiments performed in triplicates and are expressed as a fold activation to vehicle-treated sample, which was set to be 1.

*Western blotting.* Canalicular membrane vesicles were prepared as described previously using ultracentrifugation on sucrose gradient (Ito et al., 2001). Whole cell homogenate was obtained as the supernatant of tissue lysate centrifugated at 3000 ×*g*. Protein expression of respective molecules in the liver was then examined as previously described (Hirsova et al., 2013). Briefly, proteins (100 µg) were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis, transferred to a polyvinylidenedifluoride membrane (Millipore) and incubated with antibodies against Bsep (Thermo Scientific-Pierce, Cat. No. PA5-13105) or Mrp2 (Alexis, clone M2III5, Cat. No. ALX 801-037). Horseradish peroxidase-conjugated secondary antibodies were from GE Healthcare and enhanced chemiluminescence reagents were from Thermo Pierce. Densitometry was performed using ScanMaker i900 (UMAX) and QuantityOne imaging software (BioRad). Protein levels were normalized to Gapdh levels.

*qRT-PCR analysis of mRNA.* Total RNA was isolated from rat livers of Wistar rats administered 50 mg/kg/day boldine for 28 days using TRI reagent (Sigma-Aldrich, MO, USA) according to the manufacturer's protocol and converted into cDNA by a High Capacity cDNA Reverse

**Table 2**
Biliary elimination of bile acids and glutathione during saline (2.5 ml/h in Ctrl/EE groups) or boldine (50 mg/kg/h in Boldine/EE-B groups) continuous intravenous infusion to saline (Ctrl) or ethinylestradiol (EE) pretreated female Wistar rats. Biliary elimination parameters of bile acids were calculated from data measured in 0–30 min bile collection interval while those for glutathione from data measured in 90–120 min bile collection interval as stated in methods.

| | Ctrl | Boldine | EE | EE + Boldine |
|---|---|---|---|---|
| BE of BA (nmol/min/100 g BW) | 115 ± 14 | 98 ± 22 | 36 ± 6.9** | 49 ± 6.6* |
| $CL_B$ BA (ml/min/100 g BW) | 21 ± 6 | 10 ± 1.5 | 1.6 ± 0.5* | 3.3 ± 0.5* |
| BA in plasma (µM) | 8.6 ± 2.5 | 11 ± 2.6 | 35 ± 10** | 15 ± 1† |
| BE glutathione (nmol/min/100 g BW) | 14 ± 2.7 | 23 ± 2.7** | 0.3 ± 0.1*** | 0.4 ± 0.1*** |
| $CL_B$ glutathione (µl/min/100 g BW) | 39 ± 11.1 | 55 ± 6.5 | 0.8 ± 0.5*** | 1.3 ± 0.4*** |
| Glutathione in liver (nmol/mg) | 84 ± 7.1 | 73 ± 2.2 | 154 ± 6.5*** | 127 ± 7***†† |

Data are means ± SD. Abbreviations: BA – bile acids, BE – biliary excretion, $CL_B$ – biliary clearance, BW – body weight; EE – pretreatment with ethinylestradiol (5 mg/kg s.c. daily for 5 days). Significantly different from control values (*P < 0.05, **P < 0.01, ***P < 0.001).
Significantly different from EE values (†P < 0.05, ††P < 0.01).

**Table 3**
Biliary elimination of bile acids and glutathione in female TR⁻ (Mrp2-deficient) rats administered either with saline or boldine by intravenous infusion. Ctrl-L is the group of complementary female Lewis rats infused with saline. Biliary elimination parameters of bile acids were calculated from data measured in 0–30 min bile collection interval while those for glutathione from data measured in 90–120 min bile collection interval as stated in methods.

| | Ctrl-L | TR⁻ | TR⁻-B |
|---|---|---|---|
| BE of BA (nmol/min/100 g BW) | 111 ± 14 | 68 ± 4.3* | 85 ± 9 |
| $CL_B$ BA (ml/min/100 g BW) | 14 ± 2.6 | 6.9 ± 3.3 | 4.2 ± 1.1 |
| BA in plasma (μM) | 9.1 ± 1.7 | 16 ± 4.9 | 23 ± 4.1 |
| BE glutathione (nmol/min/100 g BW) | 7.7 ± 1.1 | ND | ND |
| $CL_B$ glutathione (μl/min/100 g BW) | 12 ± 1.8 | ND | ND |
| Glutathione in liver (nmol/mg) | 51 ± 2.3 | 125 ± 4.6*** | 115 ± 5.8*** |

Abbreviations: BA — bile acids, BE — biliary excretion, $CL_B$ — biliary clearance, BW — body weight, Ctrl-L — healthy Lewis rats infused i.v. with saline, TR⁻ — Mrp2-deficient Lewis rats infused i.v. with saline, TR⁻-B — Mrp2-deficient Lewis infused i.v. with boldine (50 mg/kg/h), ND — not defined because glutathione concentrations in bile were below detection limit. Data are means ± SD. Significantly different from control values (*P < 0.05. ***P < 0.001).

Transcription kit (Life Technologies, CA, USA). Gene expression was examined by quantitative real-time PCR as previously described (Hirsova et al., 2013). TaqMan Fast Universal PCR Master Mix and predesigned TaqMan Gene Expression Assay kits were purchased from Life Technologies. The glyceraldehyde 3-phosphate dehydrogenase gene was used as a reference for normalizing data.

*Immunohistochemistry of Mrp2, Bsep and ZO-1.* Serial cross-sections (7 μm) of liver median lobe taken from Wistar rats (underwent either $^{W}$I.v. infusion or $^{W}$28-day pretreatment — Table 1) were cut on a cryostat and placed on gelatin-coated slides. Five slides from each animal

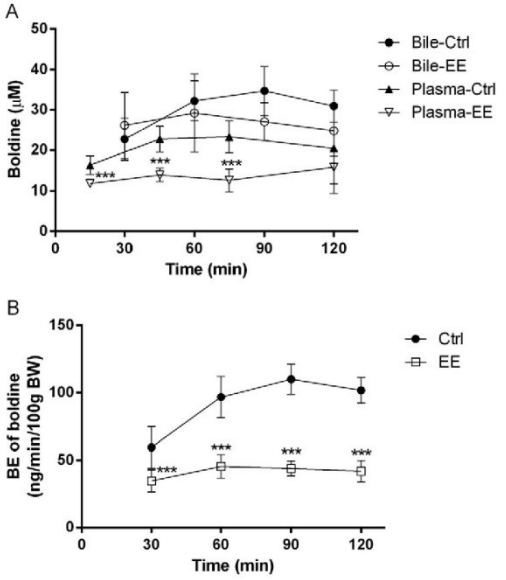


**Fig. 3.** Plasma and bile concentrations of boldine during its continuous intravenous infusion in saline (Ctrl) or ethinylestradiol (EE — 5 mg/kg/day subcutaneously for 5 days) pretreated animals (A). Pretreatment with ethinylestradiol led to a significant reduction in biliary excretion (BE) of the compound (B). Results are presented as means ± SD; significant difference from corresponding control group values (***P < 0.001).

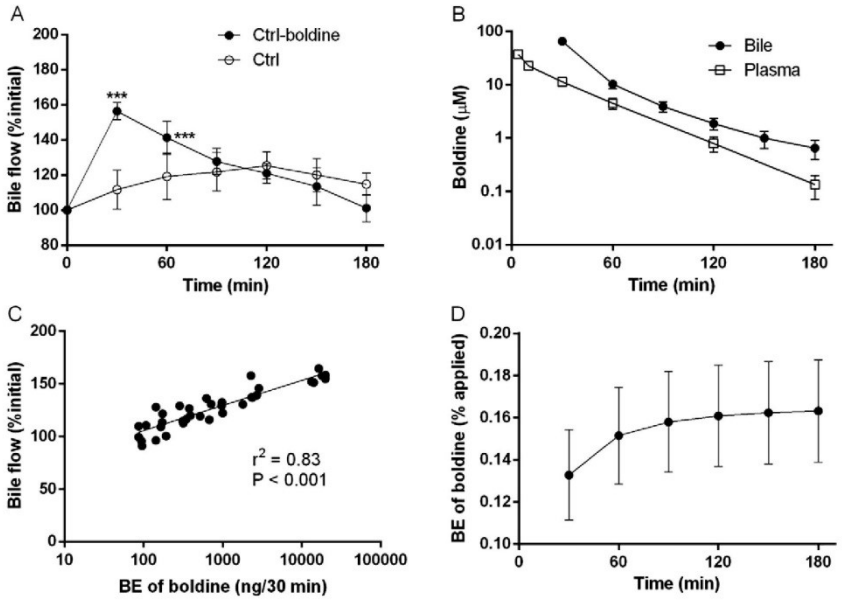


**Fig. 2.** Choleresis induced by intravenous bolus dose of boldine (50 mg/kg — A). A steep decline in plasma and biliary concentrations of boldine (B) led to a short-term choleretic effect (A) persisting for 60 min until the biliary concentrations decreased below 10 μM. A strong relationship was shown between the amount of boldine excreted into bile and the change in bile flow when compared to predose values (C). Biliary excretion of the compound over the evaluated period was less than 0.2% of the applied dose (D). Results are presented as means ± SD; significant difference from predose values of bile flow (***P < 0.001).

and from each group were taken for immunohistochemical analysis. Before antigen detection, slides were incubated with anti-avidin and anti-biotin solutions (Vector Laboratories, USA). Thereafter, slides were incubated with primary antibodies and after that with goat anti-rabbit secondary antibody marked with green fluorochrome DyLight488 (Jackson ImmunoResearch, USA) diluted 1:100 in BSA to detect ZO-1. Goat anti-mouse secondary antibody (Jackson ImmunoResearch, USA) (diluted 1:100 in BSA), goat anti-rabbit secondary antibody (Jackson ImmunoResearch, USA) (diluted 1:100 in BSA) and ExtraAvidin red fluorochrome CY3 (Sigma Chemical, USA) were used (diluted 1:300 in BSA) for the detection of either Mrp2 or Bsep. For nuclear counterstaining the blue-fluorescent DAPI nucleic acid stain (Invitrogen, Czech Republic) was used. Staining with nonimmune isotype-matched immunoglobulins assessed the specificity of the immunostaining. Primary antibodies included the following: mouse monoclonal antibody anti-Mrp2 (dilution 1:20, 1 h at RT) was purchased from Enzo Life Sciences (USA), rabbit polyclonal antibody anti-Zo-1 (dilution 1:100, 1 h at RT) was purchased from Invitrogen (USA) and rabbit polyclonal antibody anti-ABCB11 (Bsep, dilution 1:50, 1 h at RT) purchased from Termo Scientific (USA). Photo documentation and image digitizing from the microscope were performed with the Olympus AX 70, with a digital VDS Vosskühler (GmbH, Germany) with image analysis software NIS (Laboratory Imaging, Czech Republic).

*Analytical methods.* Bile acids in plasma and bile were measured using a commercial kit (Diazyme). Biochemical parameters reflecting liver

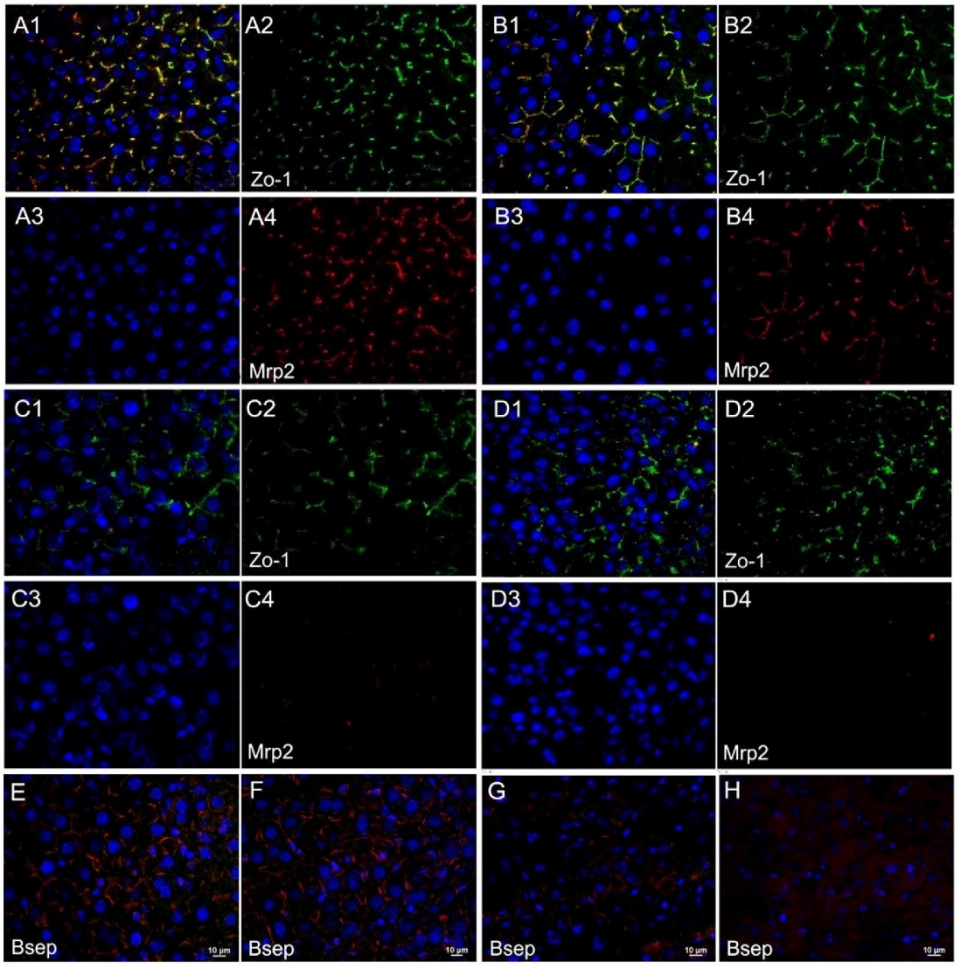


**Fig. 4.** Immunohistochemical staining of Mrp2 (red – A4, B4, C4, D4) and Bsep (red – E–H) proteins in the livers of animals pretreated either by saline (A, B, E, F) or ethinylestradiol (5 mg/kg s.c. daily for 5 days – C, D, G, H). After the pretreatment, animals received intravenous infusion of either saline (A, C, E, G) or boldine (50 mg/kg/h – B, D, F, H). Mrp2 showed strong colocalization (orange – A1, B1, C1, D1) with Zo-1 (green – A2, B2, C2, D2) protein at the canalicular membranes of hepatocytes. The nuclei were stained with DAPI (blue). Neither expression of Mrp2 (B4, D4) nor the expression of Bsep (F, H) was changed by boldine administration. Significant down-regulation of both transporters was detected in ethinylestradiol pretreated groups (C4, D4, G, H). Scale bars represent 10 µm.

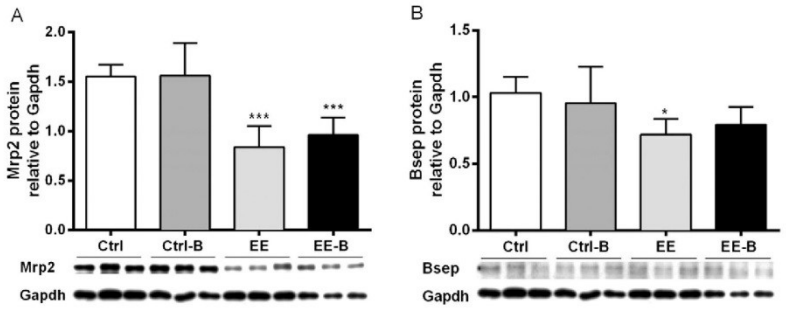


**Fig. 5.** Continuous infusion of boldine for 120 min did not change the content of Mrp2 (A) or Bsep (B) transporters at apical membranes, as measured by Western blot of the canalicular membrane vesicles. Ethinylestradiol pretreatment down-regulated both transporters at the membranes. Results are presented as means ± SD; significant difference from corresponding control values (***P < 0.001). The bars represents: white — saline infusion to healthy rats; dark gray — boldine i.v. infusion (50 mg/kg/h) to healthy rats; light gray — saline infusion to rats pretreated with 5 mg/kg/day of EE s.c. for 5 days; black — boldine i.v. infusion (50 mg/kg/h) to rats pretreated with 5 mg/kg/day of EE s.c. for 5 days.

functions were examined in plasma by routine laboratory methods on a Cobas Integra 800 (Roche Diagnostics). Boldine in plasma and bile and glutathione in bile and liver tissue were quantified by previously described HPLC methods (Kand'ar et al., 2007; Hroch et al., 2013).

*Data analyses.* Biliary clearance of glutathione was calculated as a ratio of its biliary excretion to liver tissue concentrations from the last bile collection interval (90–120 min). Biliary clearance of bile acids was calculated similarly (at the interval 0–30 min), but plasma concentrations measured at 15th minute were used instead of tissue due to inability of the used method to reliably measure liver concentrations, and the fact that the majority of bile acid for biliary secretion comes from the bloodstream upon recirculation between liver and ileum. Results are presented as mean ± SD. Comparison on multiple groups was done by one-way ANOVA followed by the Newman–Keuls post hoc test. Time-course experiments were analyzed using a two-way analysis of variance (ANOVA) followed by the Bonferroni's test for post hoc analysis. Student's *t*-test was employed for two-group comparison. Pearson correlation coefficients were used to evaluate relationships between the values. Differences were considered significant at P-value less than 0.05. All analyses were performed using GraphPad Prism 6.0 software (San Diego, USA).

## Results

*Acute effect of boldine on bile flow and biliary secretion of bile acids and glutathione*

The choleretic potential of boldine was initially characterized during its continuous intravenous infusion in healthy as well as EE challenged rats (Fig. 1A). Despite the basal bile production being markedly lowered in EE pretreated rats, boldine in both situations rapidly increased bile flow with a steady-state reached during 60 min from the start of the infusion. Constant bile production was maintained until the end of the experiment. Interestingly, infused boldine lacked any consistent effect on biliary excretion or clearance of two main osmotic constituents of bile, bile acids and glutathione (Table 2). Although an increase in biliary secretion of glutathione was detected in healthy rats, correction to its liver concentrations did not show any change in biliary clearance. Important for the sensitivity of the model was the finding that EE pretreatment, the model of intrahepatic cholestasis, markedly reduced bile excretory parameters for glutathione as well as bile acids, and increased concentrations of both in plasma and liver tissue, respectively. Interestingly, boldine in EE animals reduced plasma concentrations of BA together with tissue concentrations of glutathione despite the absence of any change in biliary excretion of these substances (Table 2).

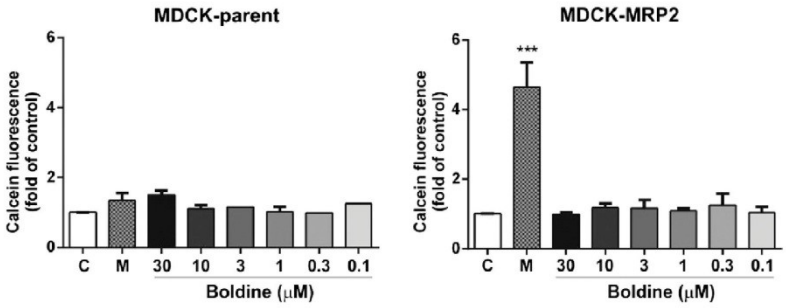


**Fig. 6.** Influence of boldine on calcein transport in parent and MRP2 expressing MDCKII cells. MK-571 (M — at concentration of 50 μM) was used as an inhibitor of MRP2 and its presence increased calcein accumulation in MRP2 expressing cells. Boldine at concentrations of 0.1–30 μM in the medium did not change calcein transport in comparison with control experiments (C — vehicle–Opti-MEM). Results are presented as means ± SD; significant differences from corresponding control values (***P < 0.001).

In order to confirm that acute choleretic activity is not associated with BAIF, we administered boldine also to Lewis TR$^-$ rats lacking Mrp2. These rats have basal bile flow reduced by 58%, and boldine was able to significantly restore this parameter to control levels within 120 min of its administration (Fig. 1B). Moreover, the choleretic effect in Lewis rats was apparent 30 min from the start of boldine's infusion similarly to situation seen in healthy and EE cholestatic Wistar rats (see Fig. 1A). Neither biliary secretion of bile acids, nor that of glutathione was modified in TR$^-$ rats by the agent (Table 3). Importantly, in the case of glutathione this absence was due to a decrease of its biliary concentrations below measurable levels in all Mrp2-negative rats, which corresponds with the nature of this animal model. Taken together, our data suggested that the acute bile flow-inducing effect of boldine is not provided by BADF or BAIF mechanisms.

*Relationship between bile formation and the kinetics of boldine excretion to bile*

The possibility that acute choleresis induced by boldine is mediated by its direct osmotic activity upon excretion into bile was tested in a situation of changing bile boldine concentrations after bolus intravenous administration. In comparison with predose values, a significant choleresis was especially apparent in the first collection interval after the injection and the effect subsided after 60 min (Fig. 2A). Consequent analysis of boldine in plasma and bile revealed a quick decline in its concentrations (Fig. 2B), which paralleled changes in the bile flow (Fig. 2A). Interestingly, during the evaluated period, less than 0.2% of the applied dose was excreted to bile (Fig. 2D). The choleretic effect of boldine was associated with its biliary concentrations of at least 10 ± 1.8 μM. As a consequence, we found a strong positive correlation ($r^2 = 0.83$, $p < 0.001$) between changes in the bile formation from the baseline values and the absolute amount of boldine excreted into the bile (Fig. 2C). To further confirm this association, we also measured plasma and bile concentrations of boldine in the specimens from control and EE-treated animals receiving continuous intravenous infusion of boldine (50 mg/kg/h — Fig. 3). In accordance with changes of bile flow (Fig. 1), plasma and bile concentrations of boldine also achieved the steady-state within 60 min of infusion (Fig. 3A). Both biliary concentrations of boldine, and the extent of its biliary excretion (Fig. 3B) were well above the threshold values for choleresis detected in intravenous bolus study.

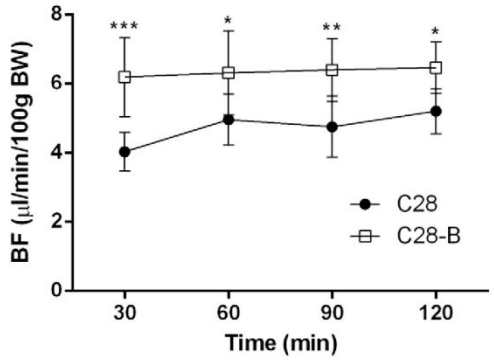


**Fig. 7.** Changes in bile production after 28-day oral pretreatment with saline (C28) or boldine (C28-B, 50 mg/kg daily) in healthy Wistar rats. Results are presented as means ± SD (n = 6–7 animals per group); significant difference from the control group (*P < 0.05, **P < 0.01, ***P < 0.001).

**Table 4**
Effect of 28-day oral pretreatment with boldine (50 mg/kg) on parameters of biliary elimination of bile acids and glutathione in female Wistar rats.

| | Ctrl | Boldine |
|---|---|---|
| BE of BA (nmol/min/100 g BW) | 102 ± 19 | 115 ± 21 |
| $CL_B$ BA (ml/min/100 g BW) | 6.2 ± 2.7 | 27 ± 5.9* |
| BA in plasma (μM) | 26 ± 6.2 | 6.6 ± 2.3* |
| BE glutathione (nmol/min/100 g BW) | 12 ± 1.9 | 12 ± 1.3 |
| $CL_B$ glutathione (μl/min/100 g BW) | 37 ± 5.5 | 42 ± 4.8 |
| Glutathione in liver (nmol/mg) | 96 ± 6.7 | 98 ± 5.8 |

Abbreviations: B — boldine pretreatment, BA — bile acids, BE — biliary excretion, BW — body weight. $CL_B$ — biliary clearance. Data are means ± SD. Significantly different from Ctrl values (*P < 0.05, **P < 0.01).

*Immunodetection of Mrp2 and Bsep in the liver after continuous infusion of boldine*

Immunohistochemistry and Western blot were used to examine changes in the liver protein content of Mrp2 and Bsep, which are rate-limiting transporters for BADF and BAIF, respectively. Double fluorescence immunohistochemical staining in the liver showed strong colocalization of Mrp2 with Zo-1, an essential protein contained in tight-junctions, at canalicular membranes of hepatocytes and verified localization of the transporter and the method reliability (Fig. 4A–D). Subsequent analysis verified also the canalicular position of Bsep (Fig. 4E–H). No apparent difference in Mrp2 or Bsep expression was detected between saline (Fig. 4A4, E) or boldine (Fig. 4B4, F) treated rats. Similarly, administration of boldine to EE-pretreated animals had no effect on the expression of Mrp2 and Bsep (Fig. 4D4, H). In support of these results, we performed Western blot analyses of canalicular membrane vesicles isolated by the ultracentrifugation on sucrose gradient (Fig. 5). No change in Mrp2 or Bsep canalicular membrane content was detected after 120 min infusion of the agent. Results of both immunodetection methods suggested that insertion of either transporter to the canalicular membrane of hepatocytes is not responsible for boldine's choleretic action.

*Effect of boldine on Mrp2-mediated transport in MDCK cells*

Boldine did not alter the transport of the selective Mrp2 substrate, calcein, in a MRP2 transfected polarized MDCK cell line (Fig. 6). It suggests that this alkaloid is not able to directly stimulate or block the MRP2 function or its localization on apical membranes of these cells. In contrast, addition of selective MRP2 inhibitor, MK-571, raised intracellular accumulation of calcein, and confirmed the sensitivity of the model. As expected, no significant change in the fluorescence was observed in the presence of boldine or MK-571 in the control MDCKII parent cells.

**Table 5**
Oral pretreatment with 50 mg/kg of boldine applied for 28 days did not change any of the evaluated biochemical parameters in the serum.

| | Ctrl | Boldine |
|---|---|---|
| Glucose (mM) | 9.0 ± 0.3 | 9.7 ± 0.4 |
| Bilirubin (μM) | 2.9 ± 0.1 | 3.0 ± 0.0 |
| ALT (μkat/l) | 1.3 ± 0.2 | 0.8 ± 0.1 |
| AST (μkat/l) | 2.5 ± 0.7 | 1.7 ± 0.2 |
| Cholesterol (mM) | 1.4 ± 0.1 | 1.4 ± 0.1 |
| Triglycerides (mM) | 1.1 ± 0.3 | 1.0 ± 0.3 |

Data are means ± SD.

*Effect of long-term oral administration of boldine on bile production*

Administration of 50 mg/kg/day boldine by gastric gavage for 28 days induced a slight but significant sustained choleresis (Fig. 7). This effect was not dependent on plasma or bile concentrations of boldine, because the bile flow measurements were performed 24-h after the last dose administration, when the agent was not measurable in these fluids. Detailed analysis of biliary excretion parameters for glutathione and bile acids was performed to identify contribution of BAIF and BADF on the observed choleresis. While no influence of boldine was detected on glutathione turnover, the compound increased biliary clearance of bile acids (Table 4). Thus, BADF seem to be primarily

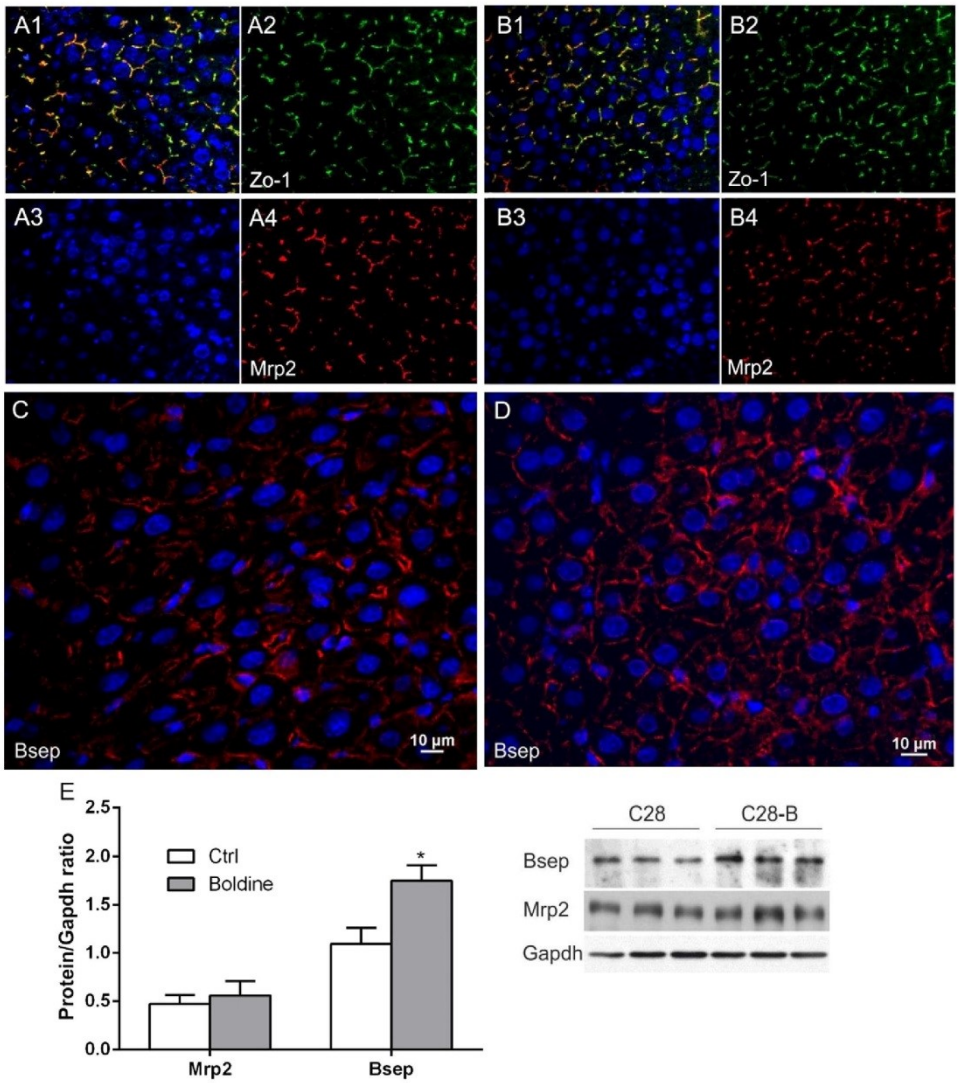


**Fig. 8.** Immunohistochemical localizations of Mrp2 (red — A4, B4) and Bsep (red — C, D) in the livers of animals that received 28-day oral pretreatment with saline (A, C) or 50 mg/kg/day of boldine (B, D). Mrp2 showed strong colocalization (orange — A1, B1) with Zo-1 (green — A2, B2) protein at the canalicular membranes of hepatocytes. The nuclei were stained with DAPI (blue). Bsep showed more intensive staining in boldine pretreated animals (D) as compared to the controls (C). In contrast, boldine pretreatment did not change the expression of Mrp2 (B4) as compared to the controls (A4). Scale bars represent 10 μm. Western blot of canalicular membrane vesicles confirmed up-regulation of Bsep protein (E). Results are presented as means ± SD; significant differences from corresponding control values (*$P < 0.05$).

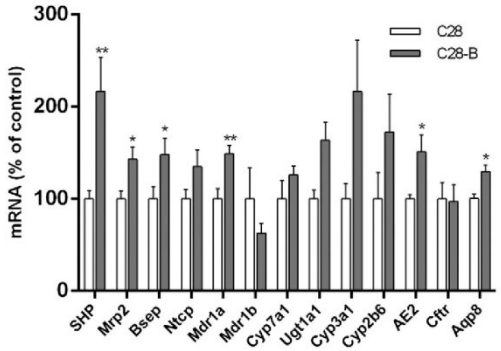


**Fig. 9.** Liver gene expression (mRNA) of the main molecules involved in bile formation. Twenty eight-day pretreatment with boldine (50 mg/day orally by gastric gavage — C28-B) led to an up-regulation of crucial transporters for bile formation. Results are presented as means ± SD; significant difference from corresponding control values (*P < 0.05, **P < 0.01).

responsible for the choleretic effect of boldine. Notably, the administration of the agent was not associated with alteration of biochemical parameters of liver injury (Table 5).

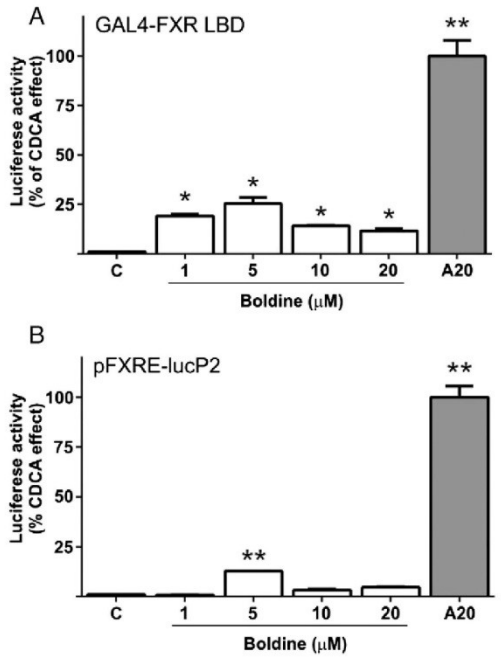


**Fig. 10.** Interaction of boldine with human FXR. The effect of different concentrations of boldine (1–20 μM) on the transient transfection gene reporter assay was performed in HepG2 cells co-transfected with the pGL5-luc luciferase reporter construct. Boldine in the medium at a concentration of 5 μM was able to activate GAL4-FXR LBD (A) and pFRE-luc2P (B) constructs. Data are shown as relative activation (mean ± SD, n = 3) of normalized luciferase activity in vehicle-treated cells. The effect of CDCA (A, 20 μM) was set to 100%. Controls (C) were incubated with vehicle only — DMSO (0.1% v/v). Significant differences compared to the control treatment: *P < 0.05, **P < 0.01.

*Immunodetection of Mrp2 and Bsep in the liver after long term-oral pretreatment with boldine*

Again, the presence of rate-limiting transporters for BA, and GSH, Mrp2 and Bsep were evaluated by immunohistochemistry and Western blot. Both methods simultaneously showed significant up-regulation of Bsep on the canalicular membrane of hepatocytes (Fig. 8) in rats administered boldine (50 mg/kg/day) for 28 days. In the same situation, no influence of boldine's pretreatment was detected on Mrp2 protein.

*Changes in liver gene expression after long-term oral administration of boldine*

The mRNA expression of the main transporters and enzymes responsible for the process of bile formation in livers taken from Wistar rats administered 50 mg/kg/day of boldine for 28 days was evaluated by real-time RT-PCR (Fig. 9). Boldine therapy increased liver mRNA of Bsep and Mrp2, but also AE2, Aqp8, Ugt1a1, and SHP transcription factor, respectively. No repression of any mRNA was detected. Because Bsep and SHP are both direct target genes for the FXR receptor, such observation suggested a transcriptional effect of boldine through this nuclear receptor.

*Effects of boldine on the Farnesoid X receptor (FXR) in HepG2 cells*

Fusion expression construct with the ligand binding domains of human FXR receptor, fused to the DNA binding domain of the yeast transcription factor GAL4 (Fig. 10A) or pFXRE-luc2P (Fig. 10B) reporter construct with FXR responsive elements of SHP gene, was used to examine the effect of boldine on FXR interaction. After 24 h treatment with increasing concentrations of boldine (1–20 μM), we observed significant activation of GAL4-FXR LBD construct by boldine at all concentrations (Fig. 10A). In case of FXRE-luc2P construct, we observed significant activation only at the concentration of 5 μM. Interestingly, higher concentrations of boldine did not activate this reporter construct significantly (Fig. 10B). Chenodeoxycholic acid (CDCA; 20 μM), a known ligand for FXR, was used as a positive control in both constructs. In summary, we suggest that boldine may exert weak activation of human FXR at physiologically relevant concentrations.

## Discussion

Choleretic activity of boldine was last evaluated by Lanhers et al. (1991) in rats who found no effect even after a bolus dose of 20 mg/kg. On the other hand, a series of older descriptive studies demonstrated significant choleretic activity of boldo extract in dogs and rats (Kreitmair, 1952; Delso-Jimeno, 1956; Bohm, 1959; Pirtkien et al., 1960; Borkowski et al., 1966; Levy-Appert-Collin and Levy, 1977). Due to growing availability of boldine-containing formulations on the market and to significant advances in the understanding of bile forming mechanisms, we decided to evaluate the acute and long-term circumstances for induction choleresis by boldine.

During continuous i.v. administration, boldine instantly raised bile production without any consistent influence on biliary excretion or clearance of glutathione or BA, indicating that BADF or BAIF was not modulated following acute administration of boldine. This finding was further supported by the absence of any difference in evaluated parameters between boldine-administered and corresponding control groups as presented using a Western blot of canalicular membrane vesicles, immunohistochemistry of liver sections, and MDCKII-MRP2 cells. Crucial in this respect was the finding that the infusion of boldine was choleretic to a similar extent in healthy animals, Mrp2-negative $TR^-$ rats and in EE-rats with a reduced expression of both Mrp2 and Bsep. Therefore, the absence of any change in BAIF or BADF suggests that the increased choleretic activity may be due to the direct osmotic activity of boldine. This hypothesis was consequently confirmed in our

single-bolus dose study where the choleretic effect of boldine tightly correlated with its changing amounts secreted into bile. The lowest biliary concentration of boldine required for induction of bile production was 10 μM. This also explains why we detected stable choleresis during continuous i.v. administration of 50 mg/kg/h when all concentrations of boldine in bile were above 13 μM. The concentration dependence was further supported by absence of choleresis during continuous infusion of less than 10 mg/kg/h of boldine (unpublished observations).

An explanation for the quick decline in boldine-induced choleresis after bolus dose lies in the rapid elimination of the agent. Jimenez and Speisky (2000) reported its very short half-life of 31 min. Although the authors did not present information about an elimination pathway, they described a markedly increased concentration of boldine within the liver when compared to brain or heart and suggested that the liver plays a pivotal role in metabolism for boldine elimination. Our recent results confirmed such a prediction demonstrating rapid *in vivo* formation of several glucuronidated and sulfated metabolites (Hroch et al., 2013). However, in the present study, we also quantified for the first time the biliary excretion of boldine, which was surprisingly low and represented less than 0.2% of the applied dose over 3 h when the rate of biliary excretion of boldine after its administration was maximal. Other elimination pathways should therefore be considered. Indeed, recently detected concentrations in urine and anticipated renal excretion of the compound were several times greater than biliary excretions (Hroch et al., 2013). However the exact contribution of this route and other extrahepatic routes to excretion of boldine from organism is currently unknown. Consequently, the capacity of liver conjugation enzymes and renal excretory pathways and their variability across different rat strains may limit the availability of boldine for biliary excretion and may explain why Lanhers et al. (1991) failed to demonstrate any choleretic effect even with a bolus i.v. dose of 20 mg/kg. Furthermore, higher biliary concentrations of boldine in comparison with plasma, and a reduction of biliary excretion in ethinylestradiol treated animals, the situation with well-described down-regulation of drug transporting proteins in the liver (Hirsova et al., 2013), suggests involvement of active transporting processes in the hepatic elimination of boldine. The molecules responsible for this process have yet to be identified however. Similarly, we are currently unable to explain the reduction of plasma bile acid concentrations in EE rats injected with boldine. A possible direct inhibitory activity of boldine on bile acid synthesis or modulation of its transport in the kidney or ileum will be a matter for further research.

In contrast to when it was administered acutely, repeated administration of 50 mg/kg/day boldine for 28 days increased bile production even though the concentration of boldine in the plasma and bile was below the limit of detection. This effect corresponded with the elevation of BA biliary clearance in parallel with transcriptional up-regulation of Bsep. We therefore analyzed relationships with the main nuclear receptor for BA, FXR, for which the Bsep is known to be one of the principal target genes (Boyer, 2013). The resultant gene reporter assay clearly showed that boldine was able to stimulate FXR nuclear receptor to an extent that corresponded with the increase in Bsep expression, BA biliary clearance and bile production. Although the stimulation of FXR was apparent mainly at 5 μM of boldine, based on its kinetics, this concentration may reliably be attained after oral administration (Jimenez and Speisky, 2000). Results of this study also suggest that boldine has the ability to stimulate FXR given evidence of increased liver mRNA of its other targets, SHP and Mrp2.

Moreover, the mRNA data showed that boldine pretreatment increased gene expression of the canalicular chloride/bicarbonate exchanger (AE2), the molecule responsible for biliary secretion of $HCO_3^-$ simultaneously with water channel aquaporin (AQP8). Induction of AE2-mediated bicarbonate rich choleresis has indeed been observed immediately after administration of secretin or glucagon as a result of increased insertion of AE2 into apical membrane of hepatocytes or cholangiocytes (Benedetti et al., 1994; Banales et al., 2006). However, the transcriptional regulation of these molecules is not known. Only van Erpecum et al. (2006) have demonstrated that AQP8 mRNA did not differ in the gallbladders of $FXR^{-/-}$ mice, which suggests that boldine modulated the transcription of both genes by a different as yet unknown mechanism. However, the resultant Western blot did not show change in the protein level of AQP8 (unpublished observation) suggesting that it is not involved in boldine's choleretic effects.

The relatively high dosage of boldine required for induction of bile production raises questions about the toxicity of the compound. Data available on its toxicity, which have been conducted mostly in isolated cells and in various animal models suggests that boldine's potential for toxicity is low (O'Brien et al., 2006). Absence of any adverse reaction was reported at doses of 50 mg/kg/day administered over 90-day period to rats (Almeida et al., 2000), which agrees with the lack of an increase in serum liver enzyme activities (AST, ALT) following boldine administration in our study. Much higher doses are required for serious toxic reactions and the reported $LD_{50}$ value for boldine was 250 mg/kg for intraperitoneal administration (Levy-Appert-Collin and Levy, 1977). Because the oral bioavailability of boldine remains to be elucidated, it is not known if the lack of toxicity observed in animals and humans was due to a lack of availability (*e.g.*, poorly absorbed) or if boldine has a low potential for toxicity. Currently, there have only been case reports of allergic reactions or increased risk of bleeding when herbal products containing boldo leaf are co-administered with anticoagulants (O'Brien et al., 2006). Lack of research in this area has led to the recent suggestion that Boldo leaf should be contraindicated especially during obstruction of the bile duct and with severe liver disease (EMEA, 2009). Our data support this recommendation because choleretic agents worsen liver damage during extrahepatic cholestasis (Fickert et al., 2002).

In conclusion, we have characterized a possible mechanism of action for boldine's choleretic activity. The agent has been found to immediately increase bile production by direct osmotic activity of the molecule itself, but should be administered in sufficient doses to attain bile concentrations of 10 μM or higher. The action of boldine is short-term given the short half-life of the agent and is independent of BADF or BAIF mechanisms. In contrast, long term pretreatment with doses of 50 mg/kg/day may induce sustained mild choleresis on the basis of FXR-mediated up-regulation of Bsep transporter with consequent stimulation of BA biliary secretion. The good safety profile of bolding is advantageous for such high-dose requirements.

## Conflict of interest statement

The authors declare they have no actual or potential conflicts of interest.

## Acknowledgment

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### 7.4. Boldine attenuates cholestasis associated with nonalcoholic fatty liver disease in hereditary hypertriglyceridemic rats fed by high-sucrose diet.

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Cílem této studie bylo objasnit vliv vysokosacharidové diety (HSD) na tvorbu žluči u potkanů s dědičnou hypertriglyceridémií (HHTg). Paralelně byl u těchto potkanů analyzován vliv boldinu, jako přírodní choleretické látky.

Vysokosacharidová dieta u HHTg potkanů vedla ke zvýšenému ukládání triglyceridů v játrech. V důsledku snížené biliární sekrece žlučových kyselin a glutathionu došlo u HHTg potkanů ke sníženému toku žluči. Odpovědným mechanismem byla down-regulace jaterních transportérů pro žlučové kyseliny a glutathion, Bsep a Mrp2. Dále byla redukována exprese genů i pro jiné transportéry podílející se na přenosu dalších složek žluči, jmenovitě Abcg5/8 pro cholesterol, Abcb4 pro fosfolipidy a Oatp1a4 pro xenobiotika. Podmínky pro rozvoj jaterního poškození kumulujícími se žlučovými kyselinami zhoršovala u aplikace HSD i indukce klíčového enzymu pro syntézu, Cyp7a1. Po aplikaci boldinu došlo částečně k oslabení cholestatického účinku HSD, především díky zvýšené expresi genů pro Bsep a Ntcp a také v důsledku zvýšené biliární sekrece glutathionu.

Závěrem můžeme říci, že tato studie popisuje mechanismy poškození toku žluči u nealkoholické steatózy jater, jako následku vysokosacharidové diety u predisponovaných jedinců. Změna v expresi transportérů může vést ke změně v kinetice léčiv a ke komplikaci při farmakoterapii u pacientů s tímto onemocněním jater. Ve studii se rovněž podařilo popsat mechanismy, kterými může přírodní choleretikum boldin zmírnit negativní dopady zvýšeného příjmu sacharózy na sekreci látek do žluči.

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# Boldine Attenuates Cholestasis Associated With Nonalcoholic Fatty Liver Disease in Hereditary Hypertriglyceridemic Rats Fed by High-Sucrose Diet

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**Summary**

The aim of the current study was to clarify the effect of high sucrose diet (HSD) on bile formation (BF) in rats with hereditary hypertriglyceridemia (HHTg). Potentially positive effects were studied for boldine, a natural choleretic agent. Administration of HSD to HHTg rats led to increased triglyceride deposition in the liver. HSD reduced BF as a consequence of decreased biliary secretion of bile acids (BA) and glutathione. Responsible mechanism was down-regulation of hepatic transporters for BA and glutathione, Bsep and Mrp2, respectively. Moreover, gene expressions of transporters for other constituents of bile, namely Abcg5/8 for cholesterol, Abcb4 for phospholipids, and Oatp1a4 for xenobiotics, were also reduced by HSD. Boldine partially attenuated cholestatic effect of HSD by promotion of biliary secretion of BA through up-regulation of Bsep and Ntcp, and by increase in biliary secretion of glutathione as a consequence of its increased hepatic disposition. This study demonstrates mechanisms of impaired BF during nonalcoholic fatty liver disease induced by HSD. Altered function of responsible transporters suggests also potential for changes in kinetics of drugs, which may complicate pharmacotherapy in subjects with high intake of sucrose, and with fatty liver disease. Sucrose induced alterations in BF may be alleviated by administration of boldine.

**Key words**

Nonalcoholic fatty liver disease • High-sucrose diet • Boldine • Bile flow • Bsep

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## Introduction

Nonalcoholic fatty liver disease (NAFLD) has become serious clinical problem affecting 30-40 % of population in some developed countries (Ali and Cusi 2009). NAFLD is closely associated with features of the metabolic syndrome such as obesity, dyslipidemia and insulin resistance (Dowman *et al.* 2010). The course of disease consist of initial stage, simple steatosis, which may last unrecognized for prolonged period, and increases vulnerability of the liver tissue to various toxic insults (Kucera *et al.* 2014). The situation may progress to more serious form of NAFLD, nonalcoholic steatohepatitis (NASH) with ongoing liver inflammation and fibrosis.

One of the mechanisms, which may contribute to increased sensitivity of liver tissue during NAFLD is the accumulation of endo-, and xenobiotics resulting from their impaired secretion into bile (Schrieber *et al.* 2008, Canet *et al.* 2015, Ferslew *et al.* 2015). The effect is ascribed to alterations in transporting proteins in the liver. Commonly described are especially upregulations of

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MRP efflux transporters at basolateral membrane of hepatocytes (Hardwick *et al.* 2011, Ferslew *et al.* 2015), downregulations of basolateral uptake transporters and variable changes of apical efflux transporters, especially Multidrug resistance-associated protein 2 (MRP2), the rate limiting for bile acid independent bile flow based on secretion of glutathione (Geier *et al.* 2005, Fisher *et al.* 2009, Canet *et al.* 2014). Clear statement about character of changes in individual transporters and definition of their clinical impact is however precluded by limited availability of human samples, by the variability between individuals, and by discrepancies between animal models used to study NAFLD (Canet *et al.* 2014). This status suggests that changes in transporting proteins may be determined by individual predisposition and by cause of NAFLD including composition of diet.

Recently, it has been stated that NAFLD with associated obesity is tightly related with increased dietary sugars income (Saab *et al.* 2015). The impact of high-sugar diet-induced NAFLD on the liver transporting proteins has not been tested yet. Several works documented that non-obese strain of hereditary hypertriglyceridemic rats (HHTg), which were selected from Wistar rats (Vrana and Kazdova 1990), may serve as suitable model of human hypertriglyceridemia (Klimes *et al.* 1995), and are very sensitive to administration of sucrose. High-sucrose diet (HSD) in this strain induces typical hallmarks of metabolic syndrome including mild weight gain, hypertension, insulin resistance with hyperinsulinemia, signs of oxidative stress (Vrana *et al.* 1993), and also increases liver weight and steatosis (Skottova *et al.* 2004). Data about liver histological status, bile formation and involved transporting processes and their modulation by HSD in this strain of rats are not available so far.

Many promising approaches exist to NAFLD therapy. One of them is stimulation of Bile salts export pump (Bsep), the rate limiting transporter for bile acid dependent bile flow (Halilbasic *et al.* 2013). The principle comes from knowledge that mice with low levels of Bsep due to absence of its main transcriptional regulator, Farnesoid X receptor (FXR), develop spontaneously hepatic steatosis, and hypertriglyceridemia with insulin resistance (Thomas *et al.* 2008, Wu *et al.* 2015). On the contrary, mice overexpressing Bsep have increased biliary lipid excretion and are protected from steatosis when fed an atherogenic diet or methionine-choline-deficient diet (Figge *et al.* 2004, Sundaram *et al.* 2005). Similar positive effect on hepatic steatosis was consequently achieved by administration of FXR receptor agonists in mice and humans (Zhang *et al.* 2009, Sanyal 2015). We have recently reported that boldine, the major alkaloid from the Chilean Boldo tree, is also agonist of FXR and produces sustained mild bile acid (BA)-dependent choleresis by upregulation of Bsep (Cermanova *et al.* 2015). Moreover, it is also effective as an antioxidant and possess significant hepatoprotective potential in various models of toxic liver injury (Lanhers *et al.* 1991, Fernandez *et al.* 2009), but its effect on NAFLD has not been tested yet. Therefore, the aim of the present work was to characterize changes in mechanisms of bile production and biliary drug excretion during NAFLD induced by high-sucrose diet in hereditary hypertriglyceridemic rats. In addition, potential for positive modulation of these changes was studied for boldine.

## Methods

*Animals and experimental design*

Two types of rats were used throughout the study: female Wistar rats (220-270 g, n=7, Velaz, Konarovice, CR) and female hereditary hypertriglyceridemic (HHTg) rats (195-300 g, n=6-7, IKEM, CR). The animals were housed under controlled environmental conditions (12-hour light-dark cycle; temperature, 22±1 °C) with a food and water freely available, and received human care according to the criteria outlined in the "Guide for the Care and Use of Laboratory Animals" published by U.S. National Institutes of health (NIH publication, 1996). The study protocol was approved by the animal welfare committee of the Charles University in Prague, Faculty of Medicine in Hradec Kralove.

HHTg rats were fed for 6 weeks with either STD (standard diet; H-S rats) or HSD (high-sucrose diet containing 50 % of sucrose; H-H rats). One group of HHTg rats received also HSD containing 0.2 % of boldine (H-H-B rats). Wistar rats fed with STD served as controls (W-S rats). The diet was isocaloric and contained equal amounts of proteins (19.6 cal%), fat (10.4 cal%), carbohydrate (70 cal%) as starch (STD) or sucrose (HSD). Bile collection study was performed in all experimental groups after overnight fasting. Rats were anesthetized with sodium pentobarbital (50 mg/kg, i.p.), fixed in a supine position on a heated platform to maintain body temperature at 37 °C, and carotid artery (for blood sampling), jugular vein (saline administration), and bile duct (for bile collection) were cannulated. All animals received continuous intravenous infusion of saline at 6 ml/h/kg to replace fluid losses by sampling.

Bile was collected in preweighted tubes at 30-min intervals over 90 min. At the end of the experiment, rats were sacrificed by exsanguination from carotid artery, and samples of serum, bile and livers were snap frozen in liquid nitrogen and stored at –80 °C until analysis.

*Serum biochemistry and bile acids and glutathione measurement*

The concentrations of glucose, bilirubin, cholesterol, HDL, TAG in serum and activities of ALT and AST in serum were measured by routine laboratory methods on Cobas Integra®800 (Roche Diagnostics, Mannheim, Germany) according to manufacturer's instructions. Bile acids (BA) in serum and bile were assayed using a commercial kit (Diazyme). Liver triglyceride concentrations were determined by commercial kits Triglycerides 250 S (Erba-Lachema s.r.o.) as described previously (Hirsova *et al.* 2012). Concentrations of reduced (GSH) and oxidized (GSSG) glutathione were analyzed separately using validated HPLC method with fluorescence detection (Hirsova *et al.* 2013).

*Quantitative real-time RT-PCR*

Gene expression was examined as previously described (Cermanova *et al.* 2014). All chemicals including TaqMan Fast Universal PCR Master Mix and pre-designed TaqMan Gene Expression Assay kits were identical with those used in our former work (Kolouchova *et al.* 2011), and all were purchased from Life Technologies. Glyceraldehyde 3-phosphate dehydrogenase (Gapdh) was used as reference for normalizing the data (Life Technologies).

*Western blot*

Crude membranes were prepared from rat liver homogenates, and were separated by SDS-PAGE electrophoresis (15 μg of protein), transferred to a PVDF membrane (Millipore) and incubated with appropriate antibodies as previously described (Hirsova *et al.* 2013). Horseradish peroxidase-conjugated secondary antibodies and enhanced chemiluminescence reagents were from GE Healthcare. The immunoreactive bands on the autoradiography films were scanned with calibrated densitometer ScanMaker i900 (UMAX, Prague, CZ) and quantified using the QuantityOne imaging software (Bio-Rad Laboratories, Hercules, CA). Expressions of proteins were normalized to β-actin levels.

*Histology*

Livers were collected immediately after death, fixed in 10 % neutral buffered formalin, embedded in paraffin, and 10 % cut to 4-5 μm thick sections. These were stained with hematoxylin-eosin and evaluated with BX-51 light microscope (Olympus) at x100 of original magnification. The liver architecture and the presence of lipid accumulation, and cellular inflammatory infiltration were assessed by the same specialist.

**Table 1.** Effect of HSD and boldine-enriched HSD on selected morphometric and serum liver biochemical parameters of HHTg rats. Wistar rats fed with STD served as controls.

| | W-S | H-S | H-H | H-H-B |
|---|---|---|---|---|
| *Glucose (mmol/l)* | 8.1 ± 1.2 | 9.3 ± 1.3 | 15 ± 5.0**†† | 14 ± 2.6**†† |
| *Bilirubin (μmol/l)* | 0.8 ± 0.5 | 1.5 ± 0.9 | 2.0 ± 0.8** | 1.4 ± 0.5 |
| *ALT (μkat/l)* | 0.7 ± 0.3 | 0.9 ± 0.2 | 0.9 ± 0.2 | 0.9 ± 0.1 |
| *AST (μkat/l)* | 1.6 ± 0.7 | 1.8 ± 0.5 | 1.6 ± 0.3 | 1.9 ± 0.5 |
| *Cholesterol (mmol/l)* | 1.1 ± 0.2 | 0.9 ± 0.2 | 1.0 ± 0.1 | 1.1 ± 0.1 |
| *HDL cholesterol (mmol/l)* | 0.9 ± 0.2 | 0.6 ± 0.2* | 0.8 ± 0.1 | 0.9 ± 0.1†† |
| *TAG (mmol/l)* | 0.3 ± 0.1 | 2.0 ± 1.6* | 2.7 ± 1.1** | 1.5 ± 1.0* |
| *BA (μmol/l)* | 3.6 ± 1.4 | 6.0 ± 1.7* | 5.5 ± 2.5* | 2.7 ± 0.7††‡ |
| *Liver weight (g)* | 7.7 ± 1.1 | 7.4 ± 1.0 | 10 ± 1.1***††† | 9.8 ± 0.7***††† |
| *Body weight (g)* | 250 ± 16.3 | 225 ± 14.0** | 284 ± 17.2***††† | 281 ± 15.7***††† |
| *Triglycerides (μmol/g liver)* | 1.6 ± 0.3 | 2.6 ± 0.8 | 4.9 ± 2.0***†† | 4.3 ± 1.6**† |

Data are presented as means ± SD from groups of 6-7 animals. W-S, control Wistar rats fed with STD; H-S, HHTg rats fed with STD; H-H, HHTg rats fed with HSD; H-H-B, HHTg rats fed with HSD enriched by 0.2 % boldine. Significant difference from W-S animals (* P<0.05, ** P<0.01, *** P<0.001); significant difference from H-S animals († P<0.05, †† P<0.01, ††† P<0.001); significant difference from H-H animals (‡ P<0.05).

*Statistical analysis*

Data are expressed as Mean ± SD. Comparison on multiple groups was done by one-way ANOVA followed by the Newman-Keuls *post-hoc* test. Differences were considered significant at $P<0.05$ value. All analyses were performed using GraphPad Prism 6.0 software (San Diego, USA).

## Results

Biochemical analysis of serum showed increased concentrations of triglycerides in all HHTg rats, compared to W-S group of rats (Table 1). Administration of HSD to HHTg rats increased serum concentrations of glucose without influence on any other evaluated biochemical parameter. HHTg animals on STD as well as on HSD presented with increased level of bile acids (BA) in serum. Addition of boldine to diet reduced bile acid serum concentrations toward levels seen in control Wistar animals on STD diet. Boldine did not change serum glucose or triglyceride concentrations.

All rats fed with HSD with or without boldine had significantly greater body and liver weights (Table 1). Histological evaluation showed normal architecture in Wistar and HHTg rats on STD (Fig. 1A/B). Addition of sucrose to diet induced significant accumulation of lipids in hepatocytes localized in pericentral region of liver lobule which presented as typical enlargement of hepatocytes and macrovesicular clarifications in the cytoplasm (Fig. 1C/D). This was in agreement with increased concentration of triglycerides in liver tissue of rats on HSD diet (Table 1). There were no signs of increased inflammatory cells infiltration induced by HSD. Boldine in diet had no influence on HSD-induced changes.

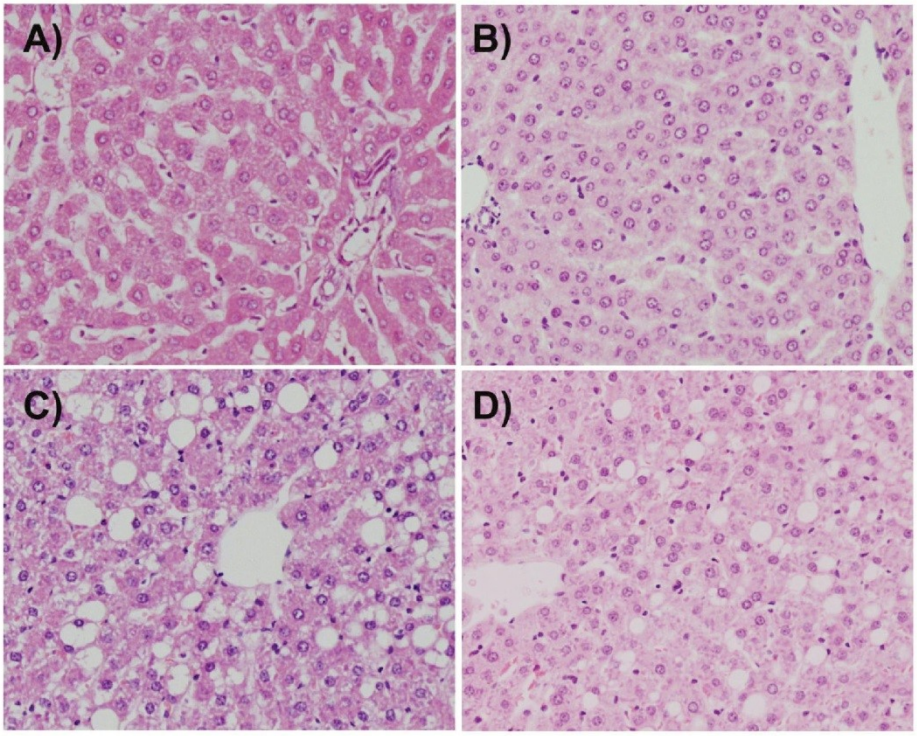


**Fig. 1.** High-sucrose diet-induced steatosis in HHTg rats. Hematoxylin and eosin-stained formalin-fixed paraffin-embedded liver sections from HHTg rats fed with HSD diet (**C**) for 6 weeks developed characteristic steatotic features with enlargement of pericentral hepatocytes, macrovesicular lipid deposits, but the absence of inflammation. Boldine did not modulate these changes (**D**). Wistar rats (**A**) and HHTg rats (**B**) fed with the control diet had healthy livers with no evidence of NAFLD. Original magnification, x100.

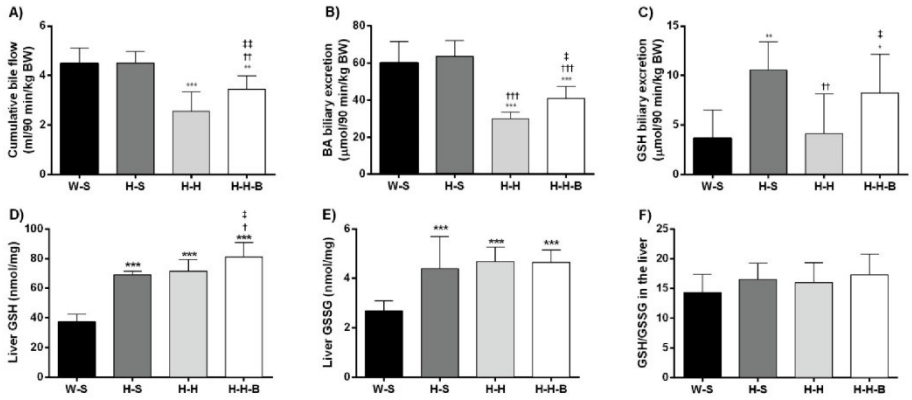


**Fig. 2.** Effect of HSD and boldine added to HSD on parameters associated with bile production in HHTg rats. Wistar rats fed with STD served as controls. Cumulative bile flow (**A**), biliary secretion of bile acids (**B**), and glutathione biliary excretion (**C**) was evaluated over 90 min. Related plasma concentrations of reduced (GSH – **D**), and oxidized (GSSG – **E**) glutathione (in nmol per mg of liver protein), and their ratio (**F**) in the liver were measured at 90$^{th}$ minute. W-S, control Wistar rats fed with STD; H-S, HHTg rats fed with STD; H-H, HHTg rats fed with HSD; H-H-B, HHTg rats fed with HSD enriched by 0.2 % boldine. Data are presented as means ± SD from groups of 6-7 animals; significant difference from W-S animals (* P<0.05, ** P<0.01, *** P<0.001); significant difference from H-S animals († P<0.05, †† P<0.01, ††† P<0.001); significant difference from H-H animals (‡ P<0.05, ‡‡ P<0.01).

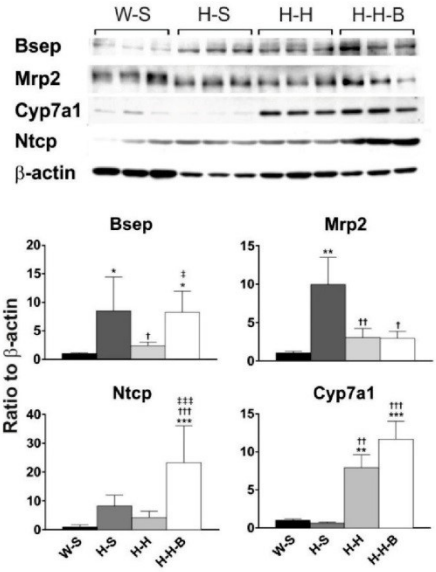


**Fig. 3.** Effect of HSD and boldine enriched HSD on the protein expression of Bsep, Mrp2, Ntcp, and Cyp7a1 in the liver of HHTg rats. Wistar rats fed with STD served as controls. W-S, control Wistar rats fed with STD; H-S, HHTg rats fed with STD; H-H, HHTg rats fed with HSD; H-H-B, HHTg rats fed with HSD enriched by 0.2 % boldine. Data are presented as means ± SD from groups of 6-7 animals; significant difference from W-S animals (* P<0.05, ** P<0.01, *** P<0.001); significant difference from H-S animals († P<0.05, †† P<0.01, ††† P<0.001); significant difference from H-H animals (‡ P<0.05, ‡‡‡ P<0.001).

HHTg rats on STD have cumulative bile flow identical with standard Wistar rats (Fig. 2). Only difference was increased biliary excretion of glutathione related to its increased concentrations in liver tissue of all HHTg rats. Administration of HSD led to significant reduction of cumulative bile flow by 43 % as a result of decreased biliary secretion of bile salts, and glutathione (Fig. 2A/B/C). Boldine significantly raised biliary secretion of both bile constituents, which resulted into increased net cumulative bile flow. Boldine also increased liver concentrations of glutathione in reduced form (Fig. 2D).

Analysis of liver gene expression of major transporters responsible for bile production showed constitutive upregulation of Oatp2 and Mrp3 mRNA and downregulation of Mrp2 in HHTg rats on STD in comparison with Wistar rats (Table 2). HSD produced downregulation of Oatp2, Ntcp, Abcg5/8, Mdr2, and Mrp3 transporters, and upregulation of Cyp7a1 mRNA. Addition of boldine to HSD increased mRNA of transporters for bile acids, Bsep, and Ntcp. In order to detect inflammatory reaction, we measured also mRNA expression of key mediators (Table 2). Interestingly, NAFLD induced by HSD diet led to paradoxical reduction of Mcp-1 and TNF-α, and boldine had no effect on these molecules.

Hepatic expression of crucial molecules for bile formation was evaluated also at protein level (Fig. 3).

Interestingly, HHTg on STD presented with increased expression of both rate limiting transporters for biliary excretion of bile acids and glutathione, Bsep and Mrp2, respectively. HSD diet downregulated both, Bsep and Mrp2, and induced protein content of Cyp7a1, the rate limiting enzyme for synthesis of bile acids from cholesterol. Addition of boldine to HSD caused upregulation of Bsep and Ntcp protein, but did not change Cyp7a1 or Mrp2 expression.

**Table 2.** Effect of HSD and boldine enriched HSD on liver mRNA expression of the main molecules involved in bile formation and regulation of inflammatory reaction in HHTg rats. Wistar rats fed with STD served as controls. W-S, control Wistar rats fed with STD; H-S, HHTg rats fed with STD; H-H, HHTg rats fed with HSD; H-H-B, HHTg rats fed with HSD enriched by 0.2 % boldine.

| Target gene | W-S | H-S | H-H | H-H-B |
|---|---|---|---|---|
| *Abcb11 (Bsep)* | 100 ± 39 | 72 ± 22 | 48 ± 17** | 93 ± 19 ‡‡ |
| *Abcc2 (Mrp2)* | 100 ± 19 | 64 ± 18*** | 51 ± 10*** | 47 ± 8*** |
| *Abcg5* | 100 ± 37 | 160 ± 105 | 23 ± 13*††† | 56 ± 39†† |
| *Abcg8* | 100 ± 59 | 96 ± 67 | 20 ± 14*†† | 33 ± 36*†† |
| *Abcb1a (Mdr1a)* | 100 ± 36 | 156 ± 76 | 104 ± 36 | 106 ± 48 |
| *Abcb1b (Mdr1b)* | 100 ± 44 | 354 ± 367 | 171 ± 190 | 63 ± 35 |
| *Abcb4 (Mdr2)* | 100 ± 25 | 114 ± 51 | 57 ± 29*† | 70 ± 16† |
| *Abcg2 (Bcrp)* | 100 ± 70 | 355 ± 105*** | 149 ± 74††† | 169 ± 61††† |
| *Slc47a2 (Mate2)* | 100 ± 49 | 159 ± 137 | 132 ± 121 | 137 ± 115 |
| *Slc10a1 (Ntcp)* | 100 ± 20 | 98 ± 18 | 72 ± 20*† | 94 ± 21 ‡ |
| *Slc22a1 (Oct1)* | 100 ± 34 | 118 ± 46 | 91 ± 22 | 91 ± 31 |
| *Slco1a4 (Oatp2)* | 100 ± 36 | 205 ± 104** | 101 ± 41†† | 99 ± 38† |
| *Slc22a7 (Oat2)* | 100 ± 31 | 99 ± 29 | 73 ± 13 | 67 ± 18 |
| *Abcc3 (Mrp3)* | 100 ± 63 | 286 ± 185** | 89 ± 29†† | 109 ± 38†† |
| *Abcc4 (Mrp4)* | 100 ± 40 | 83 ± 35 | 51 ± 21* | 53 ± 15* |
| *Cyp7a1* | 100 ± 35 | 43 ± 30 | 202 ± 73**††† | 174 ± 60*†† |
| *TGF-β1* | 100 ± 27 | 142 ± 68 | 88 ± 32* | 75 ± 22* |
| *Acta2* | 100 ± 25 | 153 ± 77 | 204 ± 101* | 144 ± 41 |
| *IL-6* | 100 ± 101 | 71 ± 60 | 32 ± 38 | 17 ± 10 |
| *Ccl2 (Mcp-1)* | 100 ± 55 | 98 ± 86 | 30 ± 40*† | 21 ± 12*† |
| *TNF-α* | 100 ± 64 | 98 ± 64 | 19 ± 27*† | 18 ± 10*†† |

Data are presented as means ± SD from groups of 6-7 animals; significant difference from W-S animals (* P<0.05, ** P<0.01, *** P<0.001); significant difference from H-S animals († P<0.05, †† P<0.01, ††† P<0.001); significant difference from H-H animals (‡ P<0.05, ‡‡ P<0.01).

## Discussion

Bile formation is a unique function of the liver which is vital to survival of the organism. Among other functions, bile is major excretory route for potentially toxic exogenous lipophilic substances including drugs, as well as for endogenous compounds such as bile salts and bilirubin (Boyer 2013). Any impairment of bile formation may therefore led to retention of such substances in the liver, where they can inflict damage, activate inflammation, fibrosis, and eventually carcinogenesis which all aggravate the underlying pathology (Cuperus *et al.* 2014). Especially bile acids are known for direct toxic effect on hepatocytes and initiation of inflammatory response in the liver, if they are retained, what can be typically seen in different type of cholestasis. Increased serum concentrations of bile acids have been recently indeed demonstrated in patients with NASH (Ferslew *et al.* 2015), which suggest that mechanism of bile formation may contribute to pathophysiology of NAFLD and that cholestasis might promote disease progression (Sorrentino *et al.* 2005). For obvious ethical reason the

bile production cannot be measured in humans. Thus available data on influence of NAFLD on bile production are scarce and are taken from different animal models.

Initial results come from obese Zucker rats and demonstrate that even simple liver steatosis without inflammation may reduce bile production as a result of decreased biliary secretion of bile acids (BA) and glutathione, the main osmotic constituents serving as driving force for bile formation. However, systemic serum concentrations of BA or bilirubin might be not affected (Pizarro *et al.* 2004, Geier *et al.* 2005). These changes were ascribed to reduced protein expression of Oatp2 (Oatp1a4), an uptake transporter for numerous endo and xenobiotic including BA, and Mrp2, the transporter for organic anions such as glutathione, bilirubin, and conjugated BA, because other transporters for BA, like Bsep or Ntcp were not affected by this model of NAFLD. Similar conclusion was presented by Kong *et al*. (2012) who showed reduced bile flow in female C57BL/6 mice fed with high-fat diet as a consequence of transcriptional downregulation of Mrp2. In contrast, other experiments with simple liver steatosis induced by high-fat diet administered in rats yielded either unchanged (Fisher *et al.* 2009) or even increased bile production (Lickteig *et al.* 2007) but results were presented without information about biliary secretion of BA or glutathione. Parallel status in protein expression of responsible transporters was absence of change in efflux Mrp2/3/4, Pgp, and Bcrp transporters, and downregulation of basolateral uptake transporters Oatp1a1/4, or Oatp1b2 (Lickteig *et al.* 2007, Fisher *et al.* 2009, Canet *et al.* 2014).

The consequence of NASH, an advanced form of NAFLD, for bile production has been characterized only in one work which showed no alteration (Lickteig *et al.* 2007). All other data focus mainly the changes in the expression of individual transporters in the liver. Commonly reported is upregulation of Mrp2/3/4 and downregulation of uptake Oatp1a1, Oatp1a4, Oatp1b2 or Ntcp at protein level (Lickteig *et al.* 2007, Cheng *et al.* 2008, Fisher *et al.* 2009, More and Slitt 2011, Canet *et al.* 2014). These results are in agreement with available human data, where the protein content of efflux MRP2/3/4/5 is induced only in NASH but not in simple steatosis. However, MRP2 function is probably hampered because of its internalization from apical membranes of hepatocytes (Hardwick *et al.* 2011). The conditions for altered bile formation are therefore met also in humans. The knowledge about BA dependent bile flow, and about protein expression of its rate limiting BSEP transporter is still missing despite described increase in BA concentrations in serum of patients with NASH (Ferslew *et al.* 2015).

Our data are highly compatible with concept of reduced bile formation in NAFLD. The experimental model based on steatosis induced by high-sucrose diet in sensitive HHTg rats was used for the first time to study relationship between NAFLD and bile formation, despite association between sugar intake, obesity and NAFLD is well known. HSD diet in HHTg rats reproduced the situation of transition between simple steatosis and NASH. We observed centrilobular and macrovesicular steatosis typical for NASH (Takahashi and Fukusato 2014) but without marks of NASH such as cellular infiltration or activation of inflammatory mediators. Compared to available data about bile production during NAFLD, we have detected more complex changes based on significant reduction of bile production as a consequence of posttranscriptional downregulation of crucial efflux proteins for biliary secretion of BA and glutathione, Bsep and Mrp2, respectively. In line with previous findings (Geier *et al.* 2005), Oatp2 uptake transporter for BA and other compounds including drugs, was also transcriptionally reduced. In addition, HSD also markedly transcriptionally increased protein expression of Cyp7a1, the rate limiting enzyme for BA synthesis. Because the main regulator of these proteins is FXR, which upon stimulation suppress expression of Cyp7a1, and induces Bsep, the changes in our study suggest that FXR activity is reduced by HSD. In agreement, recently has been described that expression of CYP7A1 is increased in obese NAFLD patients as a consequence of inhibitory effect of free fatty acids on FXR signaling (Bechmann *et al.* 2013). However, despite such complex influence on liver BA homeostasis, HSD did not further increase serum concentrations of BA, because HHTg rats on STD already presented elevated serum levels of these solutes. This effect may be related to increased expression of Mrp3, the sinusoidal efflux transporter. Absence of change in biliary excretion of BA despite upregulation of Bsep in HHTg rats on standard diet support assumption that BAs are excreted back to blood. On the other hand, reduced biliary secretion of BA in HSD animals together with their increased synthesis suggest that BA may accumulate within the steatotic liver and increase vulnerability of the tissue despite no further increase of their concentration in serum. Simultaneously, neither serum liver biochemical tests nor the expression

of proinflammatory cytokines has been changed in HHTg or HSD-HHTg rats. This effect could be ascribed to markedly higher concentration of glutathione in the liver of HHTg rats. The situation deserves further research.

Addition of boldine to HSD fed HHTg rats partly restored impaired bile production by increasing biliary secretion of both BA and glutathione. The effect on BA may be explained by moderate agonistic activity of the boldine at FXR receptor with consequent induction of Bsep (Cermanova *et al.* 2015), which in our study led to important reduction of BA levels in serum. Recently described triglyceride-lowering effect of liver FXR receptor showed in knockout mice (Schmitt *et al.* 2015) was however not achieved in our study perhaps due to low bioavailability of the compound (unpublished observation). These data comply with absence of changes in triglyceride serum concentrations after administration of similar dose of boldine in streptozotocin-treated diabetic rats (Lau *et al.* 2013). On the other hand, boldine increased liver concentrations of glutathione in reduced form. Such effect may originate from its strong antioxidant capability with proven hepatoprotective potential in various models of toxic liver injury (Lanhers *et al.* 1991, Fernandez *et al.* 2009). Because Mrp2 transporter was not changed by boldine, the stimulation of biliary excretion of glutathione may be related to its increased hepatic disposition. The mechanism of Ntcp induction by boldine has not been described yet, and requires further elucidation.

In conclusion, this study presents another model of NAFLD based on administration of high-sucrose diet to hypertriglyceridemic rats. The diet led to significant cholestasis resulting from decreased biliary secretion of bile acids and glutathione. Molecular background of these changes was downregulation of Bsep and Mrp2, and induction of Cyp7a1. The data may significantly contribute to explanation of increased serum bile acids in humans with NAFLD, and to increased sensitivity of liver tissue to endo-, and xenobiotics during NAFLD. Altered excretory function of evaluated pathways may complicate pharmacotherapy in sensitive subjects with high intake of sucrose, and with fatty liver disease. Impairment in bile production was alleviated by administration of boldine, which confirms usefulness of FXR agonists as novel therapeutic strategy for NAFLD.

## Conflict of Interest

There is no conflict of interest.

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# 8. Závěr

Eliminace léčiv z organizmu je základní podmínkou pro bezpečné používání léčiv v klinické praxi. Účastní se jí celá řada aktivních i pasivních procesů, které mohou mít za různých podmínek významně jinou kapacitu. Důsledkem je intra- a zejména interindividuální variabilita v dosahovaných plazmatických koncentracích léčiv při použití standardních dávkovacích režimů, což může podmínit selhání terapie při významném poklesu plazmatických koncentrací, nebo zejména kumulaci léčiv v organizmu s rozvojem toxicity. Z tohoto důvodu je nutné detailně studovat změny jednotlivých eliminačních drah během patologických stavů a následně použité terapie. V této souvislosti se hromadí zejména informace ukazující závažné změny ledvinných a jaterních eliminačních procesů během systémové zánětlivé reakce rozvíjející se u sepse nebo u nealkoholové steatózy jater. Naše práce proto navazovala na tyto poznatky a snažila se je rozšířit o demonstraci vlivu stávajících nebo potenciálních terapeutických přístupů.

U sepse jsme se v hodnocení změn eliminace léčiv zaměřili na terapeutické modality orientované na orgánové poškození způsobené produkovanými prozánětlivými cytokiny a následným oxidativním stresem. V detailní farmakokinetické studii se podařilo prokázat, že cílená biologická léčba antagonistou receptoru pro IL-1, anakinrou, je schopna potlačit biochemické i histopatologické známky ledvinného poškození vzniklého v důsledku septického stavu, zejména pak porušenou glomerulární filtraci. V námi sledovaných parametrech byl efekt anakinry plně srovnatelný s účinkem potentního glukokortikoidu, dexametazonu. To je plně v souladu s popsanou dominatní roli IL-1 v patofyziologii sepse (108), přičemž selektivní blokáda pouze jednoho cytokinu může být spojena s výrazně nižší incidencí metabolických nežádoucích účinků provázejících standardně používané glukokortikoidy. Práce však odhalila i úskalí použití anakinry za dané situace. Velmi důležité je zejména vhodné načasovaní dávkování s ohledem na nutnost udržovat stálé koncentrace léčiva v kritické iniciální fázi septického stavu, kdy je produkce prozánětlivého IL-1 beta nejvyšší (109). U tohoto léčiva vykazuje potkan proti člověku několikanásobně rychlejší eliminaci. Zjistili jsme, že účinku anakinry lze za takových podmínek dosáhnout pouze podáváním v intervalu po 5 hodinách namísto 1x za den, což patrně vysvětluje diskrepance ve výsledcích předešlých studií (81). Druhým limitujícím faktorem je menší vliv selektivní blokády IL-1 receptoru na tubulární sekreční funkce v porovnání s dexametazonem. Tento efekt ukazuje na spolupodíl dalších mediátorů na regulaci změn renálních tubulárních funkcí během sepse a potvrzuje komplexnější účinek kortikosteroidů na jednotlivé mechanismy zánětu. Naproti tomu paralelní intrahepatální

cholestáza provázející podání LPS byla oběma léčivy ovlivněna podobně. Jak dexametazon, tak i anakinra zlepšily všechny sledované bioindikátory jaterního poškození. IL-1 beta má tedy během sepse majoritní roli v rozvoji jaterní patologie. Naše data tak otevírají možnost cíleného řešení této situace v klinických aplikacích prostřednictvím blokády receptorů pro IL-1.

Chelatace železa se jevila jako další možnost omezení rozvíjejícího se tkáňového poškození. V sérii prací se ukázalo, že krátkodobá chelatace železa během sepse snižuje produkci cytokinů a dokonce zlepšuje přežívání (110). Současně nebylo známo, jak se liší efekt mezi chelatací exctracelulární a intracelulární. Proto byl v naší následující studii hodnocen potenciálně protektivním vlivem dvou chelátorů železa, deferoxaminu (DFO, aktivní převážně extracelulárně) a dexrazoxanu (DEX, aktivní převážně intracelulárně), kde unikátnost přístupu spočívala jak v hodnocení eliminačních funkcí jater, tak v použití intracelulárně působícího DEX. Jaterní patologie byla i zde vyvolána modelovým septickým stavem navozeným u potkanů podáním endotoxinu. Nejdůležitější bylo zjištění, že obě látky vykazují v prevenci jaterního poškození diametrálně odlišné výsledky, a to navzdory podobnému účinku na snížení obsahu železa v játrech, a na potlačení produkce hlavních prozánětlivých cytokinů. Dle všech sledovaných indikátorů působil hepatoprotektivně pouze DFO, zatímco DEX poškození jater v některých parametrech signifikantně zhoršoval – zejména se jednalo o parametry oxidačního stresu charakterizovaného poměrem redukované a oxidované formy glulathionu. Tuto diskrepanci lze vysvětlit rozdíly v mechanismech účinku obou chelátorů. DFO vykazuje kromě přímého chelatačního účinku, který rychle mění dostupnost železa pro buňky imunitního systému i přímou zhášecí aktivitu na reaktivní formy kyslíku (111). Naopak DEX vyžaduje intracelulární bioaktivaci na aktivní ADR-925 metabolit, který může vytvořit komplex s volným železem [Fe(ADR-925) $H_2O]^+$, který obsahuje labilní molekulu vody (112). Tento komplex je redoxně nestabilní a může podpořit rozvoj oxidačního stresu ve tkáních. Z těchto dat lze tedy uzavřít, že prostá chelatace železa může být velmi podpůrným faktorem pro prevenci poškození jater během sepse, nicméně individuální charakteristiky jednotlivých chelátorů mohu tento efekt dále modifikovat. Pro další studie je jednoznačně příznivější použití deferoxaminu.

Dalším originálním výsledkem studie s podáním chelátorů u potkanů byla detekce poklesu genové exprese vybraných základních transportních proteinů účastnících se eliminace látek do žluče po obou podaných chelátorech u zdravých zvířat. DFO vykazoval tento efekt i u zvířat s aplikovaným LPS. To znamená, že nedostatek železa má podíl na regulaci exprese

těchto transportérů a teoreticky může měnit kinetiku jejich substrátů. Jelikož v dané oblasti neexistují odpovídající studie, stala se tato data podkladem pro další práci týmu, protože deficit železa je jedním z velmi častých klinických problémů, který postihuje až 25 % světové populace.

Jiným přístupem, jak příznivě ovlivnit stavy spojené s intrahepatální cholestázou je použití choleretik s protizánětlivým účinkem. V této oblasti jsme proto věnovali pozornost látce splňující obě kritéria, alkaloidu boldinu. Jelikož u této látky nejsou k dispozici data demonstrující princip jejího choleretického účinku, musel se tak studovat v plné šíři. Podstatou tvorby žluči je transportéry zprostředkovaná biliární sekrece osmoticky aktivních látek vykonávaná hepatocyty a cholangiocyty. Rozlišují se dvě hlavní cesty tvorby žluči: na žlučových kyselinách (BA) závislá (BADF) a na žlučových kyselinách nezávislá (BAIF) tvorba žluči. Zatímco BADF zahrnuje mechanismy syntézy a transportu výlučně žlučových kyselin, v BAIF je významná biliární exkrece několika chemicky různých látek (např. $HCO_3$), především však glutathionu (GSH), který se dostává do žluče činností Mrp2 transportéru. Tato role glutathionu navazuje na jeho významnou úlohu v antioxidační obraně, která chrání hepatocyty před poškozením kyslíkovými radikály a elektrofily (113).

Choleretika jsou látky, které stimulují tvorbu žluči aktivací BAIF/BADF nebo při jejich vysoké koncentraci ve žluči mohou přímo působit vlastní osmotickou aktivitu v závislosti na dosažené koncentraci. Hlavním cílem provedené studie bylo proto zjistit podmínky, které jsou potřebné pro choleretický efekt boldinu, jak u zdravých potkanů, tak u potkanů s poškozeným BADF/BAIF mechanismem po aplikaci EE (ethinylestradiol) nebo u potkanů s vrozeným nedostatkem Mrp2 transportérů. Pro zjištění mechanismu, který zodpovídá za choleretický efekt boldinu, jsme analyzovali jak na BA závislou, tak nezávislou tvorbu žluči, a to buď po bolusu nebo kontinuální *i.v.* aplikaci (akutní efekt) nebo po 28-ti denní premedikaci *p.o.* (dlouhodobý účinek). Během kontinuální *i.v.* aplikace boldin zvýšil produkci žluče bez ovlivnění biliární exkrece nebo clearance glutathionu a žlučových kyselin. To znamená, že BADF a BAIF nebyly během akutního podání boldinu modulovány. V této souvislosti bylo důležité zjištění, že choleretický efekt boldinu byl srovnatelný u kontrolní skupiny, Mrp2 negativní a u skupiny s EE, kdy došlo k downregulaci exprese transportérů Mrp2 a Bsep. To ukazuje, že zvýšená choleretická aktivita byla způsobena přímou osmotickou aktivitou boldinu. Tato hypotéza byla následně potvrzena po bolusové samostatné dávce boldinu, kdy jeho choleretický efekt úzce koreloval se změnami množství secernovaného do žluči. Rychlý úbytek

boldinem (bolus) navozené cholerézy můžeme přičíst rychlé eliminaci této látky, což bylo následně potvrzeno v naší recentní studii (114).

Zvýšenou tvorbu žluči způsobilo i opakované podání boldinu po dobu 28 dnů, přestože koncentrace boldinu v plazmě a žluči byla pod detekovateným limitem. Tento efekt souvisí se zvýšenou biliární clearance žlučových kyselin a s up-regulací exprese Bsep. Za použití gene-reporter assaye v kombinaci s výsledky *in vivo* aplikace bylo zjištěno, že boldin je schopný stimulovat nukleární receptor FXR, který je hlavním receptorem pro žlučové kyseliny a Bsep je jeden z jeho hlavních cílových genů. Kromě Bsep se zvýšila exprese mRNA i dalších FXR cílových genů, SHP a Mrp2. Dále premedikace boldinem zvýšila expresi kanalikulárního AE2 (chloride/bicarbonate exchanger), tj. molekuly, která je zodpovědná za biliární sekreci $HCO_3^-$ zároveň s Aqp8 (water channel aquaporin). V této studii se tedy podařilo poměrně komplexně charakterizovat mechanismus choleretického efektu boldinu. Tato látka ihned po aplikaci zvyšuje biliární produkci přímou osmotickou aktivitou, ale musí být podávána v dostatečné dávce, aby dosáhla koncentrace ve žluči více než 10 μM. Naopak dlouhodobé podávání boldinu v dávce 50 mg/kg/den může vyvolat dlouhodobě zvýšenou cholerézu založenou na up-regulaci Bsep transportéru prostřednictvím stimulace FXR nukleárního receptoru. Důsledkem pro praktické používání boldinu jako potravinového doplňku mohou být potenciální interakce s léčivy využívajícími transportní proteiny řízené FXR receptorem, kdy lze předpokládat urychlenou eliminaci.

Cílem další studie bylo objasnit účinek vysokosacharidové diety (HSD) na tvorbu žluči u potkanů s dědičnou hypertriglyceridémií (HHTg) a potenciálně pozitivní vliv přírodního choleretika boldinu. NAFLD (nonalcoholic fatty liver disease) je závažným onemocněním, které postihuje 30-40 % populace ve vyspělých zemích. NAFLD je úzce spojena se znaky metabolického syndromu, jako je obezita, dislipidemie a inzulinová rezistence. Průběh onemocnění doprovází steatóza a zvyšuje se citlivost jaterní tkáně na různé toxické podněty. Jedním z mechanismů, které ke vzniku steatózy přispívají, je porucha sekrece endogenních a exogenních látek do žluče a s tím související následná akumulace těchto látek. Tento efekt je připisován změnám v jaterních transportních proteinech. Popisovány jsou změny exprese především up-regulace Mrp efluxních transportérů na bazolaterální straně hepatocytů, downregulace transportérů pro vychytávání na bazolaterální straně a různé změny v expresi apikálních efluxních transportérů, kam patří Mrp2. V souladu s těmito změnami jsou u nemocných s NAFLD popisovány zvýšené koncentrace žlučových kyselin, které jsou pak

jedním z hlavních nox podporujících poškození jater a progresi do vážnějších stádií onemocnění.

Námi zjištěná data odpovídají konceptu snížené tvorby žluče u NAFLD. Byl použit experimentální model zvířat založený na steatóze, která byla vyvolaná HSD u HHTg potkanů. V dané souvislosti bylo využití tohoto modelu průkopnické, jelikož většina modelů používá čistě vysokolipidové diety. Nicméně z populačních studií je známo, že za metabolický syndrom a rozvoj závažnějších forem NAFLD je významně spoluzodpovědný zejména zvýšený příjem cukrů (115). V souladu s tím vykazovala zvířata s HSD významné snížení produkce žluče v důsledku posttranskripční downregulace hlavních efluxních transportérů pro biliární sekreci žlučových kyselin a glutathionu, Bsep a Mrp2. Kromě toho, vysokosacharidová dieta výrazně zvýšila expresi proteinu Cyp7a1, který je rychlost-určujícím enzymem pro syntézu žlučových kyselin. Redukovaná biliární exkrece žlučových kyselin u HSD společně se zvýšenou syntézou žlučových kyselin naznačuje, že se žlučové kyseliny mohou akumulovat ve steatických játrech a dále zvyšovat zranitelnost tohoto orgánu. Perorální podávání boldinu HHTg potkanům s vysokosacharidovou dietou částečně obnovilo sníženou tvorbu žluči zvýšením biliární sekrece, jak žlučových kyselin, tak glutathionu. Účinek boldinu na žlučové kyseliny lze vysvětlit mírnou aktivací FXR receptoru a následnou indukcí Bsep a cholerézou spojenou s významným snížením hladiny žlučových kyselin v séru. Závěrem lze říci, že vysokosacharidová dieta může u jedinců se sklonem ke zvýšeným hladinám triglyceridů v séru vést k cholestáze v důsledku snížené biliární sekrece žlučových kyselin a glutathionu. Molekulárním mechanismem těchto změn byla downregulace Bsep a Mrp2, a indukce Cyp7a1. Jelikož jsou tyto transportéry rovněž důležité v kinetice léčiv, pozorované změny mohou mít vliv na farmakoterapii u jedinců s vysokým příjmem sacharidů a se steatózou jater. Po podání boldinu byla částečně obnovena porucha produkce žluči, což potvrzuje důležitost FXR agonistů jako nové terapeutické modality v léčbě NAFLD.

## 9. Seznam doposud publikovaných prací kandidátky v odborných časopisech s impaktem

1. **IL-1 receptor blockade alleviates endotoxin-mediated impairment of renal drug excretory functions in rats.**

Kadova Z, Dolezelova E, Cermanova J, Hroch M, Laho T, Muchova L, Staud F, Vitek L, Mokry J, Chladek J, Havlinova Z, Holecek M, Micuda S

Am J Physiol Renal Physiol. 2015 Mar 1;308(5):F388-99

$IF_{(2015)}$ 3,3

2. **Deferoxamine but not dexrazoxane alleviates liver injury induced by endotoxemia in rats.**

Cermanova J, Kadova Z, Dolezelova E, Zagorova M, Safka V, Hroch M, Laho T, Holeckova M, Mokry J, Kovarikova P, Bures J, Sterba M, Micuda S.

Shock. 2014 Oct;42(4):372-9.

$IF_{(2014)}$ 2,732

3. **Boldine enhances bile production in rats via osmotic and farnesoid X receptor dependent mechanisms.**

Cermanova J, Kadova Z, Zagorova M, Hroch M, Tomsik P, Nachtigal P, Kudlackova Z, Pavek P, Dubecka M, Ceckova M, Staud F, Laho T, Micuda S.

Toxicol Appl Pharmacol. 2015 May 15;285(1):12-22.

$IF_{(2015)}$ 3,633

4. **Boldine attenuates cholestasis associated with nonalcoholic fatty liver disease in hereditary hypertriglyceridemic rats fed by high-sucrose diet.**

Zagorova M, Prasnicka A, Kadova Z, Dolezelova E, Kazdova L, Cermanova J, Rozkydalova L, Hroch M, Mokry J, Micuda S

Physiol Res. 2015;64 Suppl 4:S467-76

$IF_{(2015)}$ 1,643

5. **Epigallocatechin gallate enhances biliary cholesterol secretion in healthy rats and lowers plasma and liver cholesterol in ethinylestradiol-treated rats.**

Hirsova P, Kolouchova G, Dolezelova E, Cermanova J, Hyspler R, Kadova Z, Micuda S.

Eur J Pharmacol. 2012 Sep 15;691(1-3):38-45.

$IF_{(2012)}$ 2,516

6. **Cholestatic effect of epigallocatechin gallate in rats is mediated via decreased expression of Mrp2.**

Hirsova P, Karlasova G, Dolezelova E, Cermanova J, Zagorova M, Kadova Z, Hroch M, Sispera L, Tomsik P, Lenicek M, Vitek L, Pavek P, Kucera O, Cervinkova Z, Micuda S.

Toxicology. 2013 Jan 7;303:9-15.

$IF_{(2013)}$ 3,681

7. **Boldine Inhibits Mouse Mammary Carcinoma In Vivo and Human MCF-7 Breast Cancer Cells In Vitro.**

Tomsik P, Micuda S, Muthna D, Cermakova E, Havelek R, Rudolf E, Hroch M, Kadova Z, Rezacova M, Cmielova J, Zivny P.

Planta Med. 2016 Nov;82(16):1416-1424.

$IF_{(2016)}$ 1,99

## 10. Seznam odborných publikací prezentovaných na konferencích

**3. Postgraduální a 1. postdoktorandská vědecká konference FaF 2013**

IL-1 receptor blockade alleviates endotoxin-mediated impairment of renal drug excretory functions in rats.

*Kadova Z., Dolezalova E., Cermanova J., Hirsova P., Fuksa L., Hroch M., Zagorova M., Staud F., Micuda S.*

**4. Postgraduální a 2. postdoktorandská vědecká konference FaF 2014**

Endotoxin-mediated impairment of renal drug excretory functions in rats is prevented by two anti-inflammatory agents, dexamethasone and IL-1 receptor antagonist, anakinra.

*Kadova Z., Dolezelova E., Cermanova J., Fuksa L., Hroch M., Zagorova M., Staud F., Micuda S.*

**5. Postgraduální a 6. postdoktorandská vědecká konference FaF 2015**

Il-1 receptor blockade alleviates endotoxin-mediated impairment of renal drug excretory functions in rats

*Kadova Z., Dolezelova E., Cermanova J., Staud F., Micuda S.*

**63. Farmakologické dny Olomouc – 2013**

Boldine enhances bile production in rats

*Zagorova M., Kadova Z., Hroch M., Cermanova J., Hajkova J., Tomsik P., Kudlackova Z., Nachtigal P., Pavek P., Ceckova M., Staud F., Mokry J., Micuda S.*

**64. Farmakologické dni Martin – 2014**

Choleretic effect of boldine

*Zagorova M., Kadova Z., Hroch M., Cermanova J., Hajkova J., Pavel Tomšík, Kudlackova Z., Ceckova M., Staud F., Nachtigal P., Pavek P., Mokry J., Micuda S.*

**65. Farmakologické dny Praha – 2015**

Boldine attenuates cholestasis associated with nonalcoholic fatty liver disease in hereditary hypertriglyceridemic rats fed by high sucrose diet

*Zagorova M., Prasnicka A., Kadova Z., Dolezelova E., Kazdova L., Cermanova J., Rozkydalova L., Hajkova J., Mokrý J., Micuda S.*

**66. Farmakologické dny Brno – 2016**

Důsledky deficitu Mrp2 pro farmakokinetiku boldinu u potkana

*Cermanova J., Prasnicka A., Kadova Z., Dolezelova E., Rozkydalova L., Tomsik P., Hroch M., Micuda S.*