Univerzita Karlova v Praze
Farmaceutická fakulta v Hradci Králové
Katedra farmakologie a toxikologie

VÝZNAM TRANSPORTNÍCH PROTEINŮ A BIOTRANSFORMAČNÍCH ENZYMŮ PRO OCHRANNOU FUNKCI PLACENTY

Disertační práce

Mgr. Zuzana Vacková

Obor: Farmakologie

Školitel: Doc. PharmDr. František Štaud, PhD.

Hradec Králové 2009

Prohlašuji, že tato práce je mým původním autorským dílem, které jsem vypracovala samostatně. Veškerá literatura a další zdroje, z nichž jsem při zpracování čerpala, jsou uvedeny v seznamu literatury a v práci řádně citovány.

Poděkování

Ráda bych na tomto místě poděkovala všem, kteří mě během studia podporovali a významně tak přispěli ke vzniku této práce.

Na prvním místě bych chtěla vyjádřit velký dík mému školiteli Doc. PharmDr. Františku Štaudovi, PhD. za kritické připomínky i cenné rady a především za jeho neobvykle přátelský a otevřený přístup k odbornému vedení mé práce.

Dále bych ráda poděkovala mým kolegům z laboratoře PharmDr. Martině Čečkové, PhD. a Doc. PharmDr. Petru Pávkovi, PhD., kteří mi byli vždy ochotně nápomocní při řešení praktických i teoretických problémů, se kterými jsem se během studia potýkala. Neocenitelná byla i pomoc a podnětné rady PharmDr. Antonína Libry, PhD. vztahující se k metodice real-time RT-PCR. Poděkování náleží i všem ostatním kolegům a přátelům z katedry farmakologie a toxikologie za vřelé přijetí do jejich kolektivu a vytvoření velmi příjemných pracovních podmínek.

Děkuji také Prof. RNDr. Jiřímu Páchovi, DrSc., RNDr. Karle Vágnerové, PhD. a dalším členům výzkumného týmu z Fyziologického ústavu Akademie věd České republiky za odborné konzultace a aktivní spolupráci na řešených projektech.

Na závěr bych chtěla poděkovat mým rodičům, bez jejichž počáteční iniciativy by tato práce nikdy nevznikla, za jejich trpělivost, laskavost a podporu, kterou mi během celého studia projevovali.

Seznam zkratek

11β-HSD	11β-hydroxysteroid dehydrogenáza
ABC	"ATP binding cassette" - skupina transportérů vázajících ATP
BCRP	"breast cancer resistance protein"
CYP450	cytochrom P450
GK	glukokortikoidy
MDR	"multidrug resistance protein"
MRP	"multidrug resistance related protein"
NBD	"nucleotide binding domain" - nukleotidy vázající doména
P-gp	P-glykoprotein
RT-PCR	"reverse transcription - polymerase chain reaction" - reverzní transkripce - polymerázová řetězová reakce
TMB	"transmembrane domain" - transmembránová doména

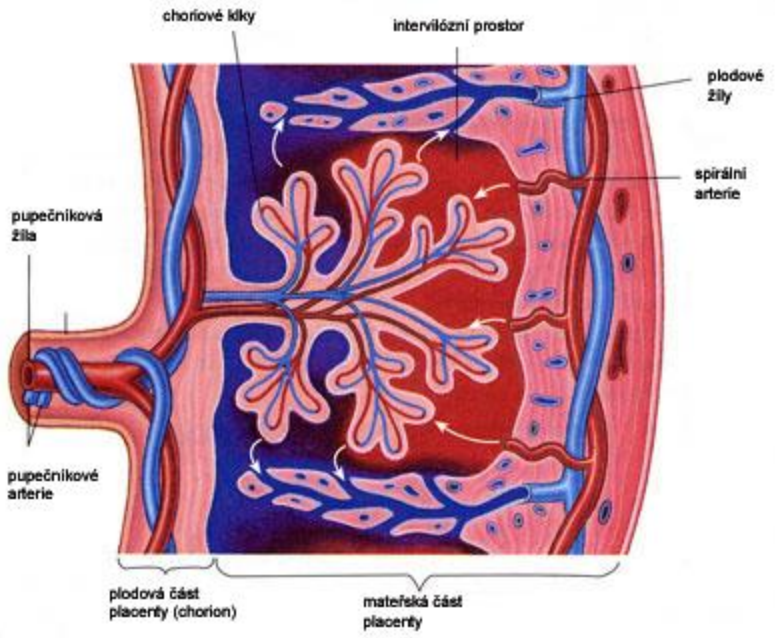
Obsah

I.
ÚVOD A CÍLE PRÁCE

1. Struktura a funkce placenty

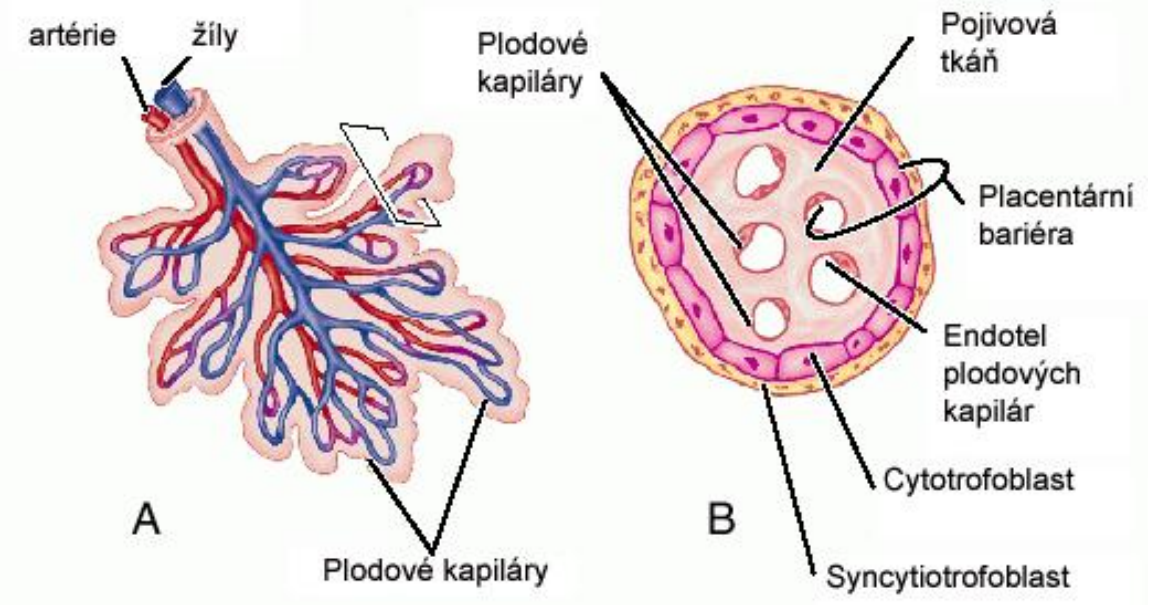
Placenta je specifický orgán mající tvar disku s charakteristickou lobulární strukturou, který přivádí do těsné blízkosti krevní oběh matky a plodu a zároveň vytváří mezi oběma krevními řečišti bariéru tak, že nedochází ke vzájemnému míšení krve. Placenta plní celou řadu vitálních funkcí, které jsou nezbytné pro správný vývoj plodu. Jedná se především o zajištění přísunu živin a kyslíku a současnou exkreci zplodin metabolismu. Vedle transportu látek probíhá v placentě syntéza celé řady hormonů, enzymů, regulačních faktorů a signálních molekul, které významně ovlivňují celkový průběh těhotenství.

Základní struktura lidské placenty (Obr. 1) vzniká jak z mateřské tkáně - decidua bazalis, tak z tkáně plodové - chorion frondosum. Zralá placenta je funkčně rozdělena na 20 - 40 kotyledonů oddělených od sebe septy vybíhajícími z decidua bazalis. V každém z kotyledonů se nachází tzv. **choriové klky** (Obr. 2, A) tvořené pojivovou tkání a hustou sítí kapilár, ve kterých proudí krev plodu přiváděná a odváděná pupečníkovými cévami. Povrch choriových klků je pokryt vrstvou trofoblastu, který je v přímém kontaktu s mateřskou krví přiváděnou spirálními arteriemi (Panigel, 1986; Enders and Blankenship, 1999).



Obr. 1 *Schématické znázornění struktury placenty. Převzato a upraveno z: www.biog1105-1106.org.*

Podle počtu buněčných vrstev tvořících bariéru mezi mateřskou a fetální krví lze savčí placenty klasifikovat do tří skupin: a) typ hemochoriální (člověk, potkan, králík), b) typ endotheliochoriální (kočka, pes) a c) typ epiteliochoriální (ovce, prase, kůň). V lidské, hemochoriální, placentě tvoří bariéru oddělující krevní řečiště matky a plodu dvě buněčné vrstvy: **trofoblast** a **endotel fetálních kapilár** (Obr. 2, B). Na začátku vývoje placenty tvoří trofoblast především mitoticky aktivní jednobuněčný cytotrofoblast, který později fúzuje v mnohojaderný syncytiotrofoblast - hlavní složku placentární bariéry. Se vznikem syncytiotrofoblastu je spojeno i postupné zmenšování tloušťky placentární bariéry z počátečních 50 - 100 μm až na pouhých 4 - 5 μm v pozdních stádiích těhotenství.



Obr. 2 *Schématické znázornění placentární bariéry.* ***A*** *– choriový klk a síť fetálních kapilár,* ***B*** *– vlastní placentární bariéra. Převzato a upraveno z: www. medical-dictionary.thefreedictionary.com*

Plazmatická membrána syncytiotrofoblastu je polarizovaná. Na apikální membráně (orientované na mateřskou stranu) vytváří tzv. kartáčový lem s množstvím mikroklků, které jsou omývány mateřskou krví. Kartáčový lem výrazně zvětšuje absorpční povrch trofoblastu. Bazální membrána (orientovaná na plodovou stranu) tyto mikroklky postrádá. Polarizace syncytiotrofoblastu je dále umocněna rozdílnou distribucí proteinů, především enzymů, receptorů a transportních proteinů, na obou stranách membrány (Syme et al., 2004; Ganapathy and Prasad, 2005).

2. Ochranná funkce placenty

Jednou z významných fyziologických funkcí placenty je ochrana plodu proti potenciálně toxickým látkám přítomným v mateřské krvi. Vedle polutantů, toxinů či návykových látek, jsou to především léčiva, která jsou často během těhotenství podávaná za účelem terapie matky (infekční onemocnění, hypertenze, diabetes mellitus, epilepsie apod.) nebo plodu (antivirotika – omezení přenosu HIV, fetální tachykardie aj.). Převážná většina látek prochází přes placentární bariéru prostou difúzí na základě koncentračního gradientu. Faktory, které limitují rychlost tohoto průchodu jsou především: fyzikálně-chemické vlastnosti dané molekuly (molekulová hmotnost, lipofilita, náboj, interakce s plazmatickými bílkovinami) a vlastnosti placentární bariéry (průtok krve placentou, velikost absorpčního povrchu, tloušťka bariéry, pH). Molekuly cizorodých látek mohou být také rozpoznávány jako substráty některých membránových přenašečů pro endogenní látky lokalizovaných na membránách syncytiotrofoblastu a endotelu fetálních kapilár. Transport těmito přenašeči probíhá buď usnadněnou difúzí nebo aktivním transportem za spotřeby energie (Ganapathy and Prasad, 2005).

Ochrannou funkci placenty zajišťuje existence placentární bariéry, která je tvořena dvěma hlavními složkami - **mechanickou a aktivní.** Podstatou mechanické bariéry v hemochoriální placentě jsou dvě buněčné vrstvy oddělující krevní řečiště matky a plodu - **mnohojaderný syncytiotrofoblast** a **endotel fetálních kapilár**. Podobně, jako je tomu v jiných fyziologických bariérách (např. hemato-testikulární, hemato-mozková apod.) jsou i v placentě tyto buněčné vrstvy organizovány tak, že umožňují pouze intracelulární průchod látek z jednoho kompartmentu do druhého. V placentě tak každá látka při transportu z krve matky do krve plodu musí překonat nejprve apikální a bazální membránu syncytiotrofoblastu a následně endotel kapilár (Obr. 2, B).

Aktivní složka placentární bariéry je realizována dvěma hlavními mechanismy: a) **aktivitou efluxních lékových transportérů** a b) **aktivitou biotransformačních enzymů** lokalizovaných v placentární bariéře. První z uvedených mechanismů má pravděpodobně z hlediska ochrany plodu převažující význam, nicméně, i relativně bohatá metabolická aktivita probíhající v placentě zřejmě značně ovlivňuje průchod řady exogenních i endogenních látek přes

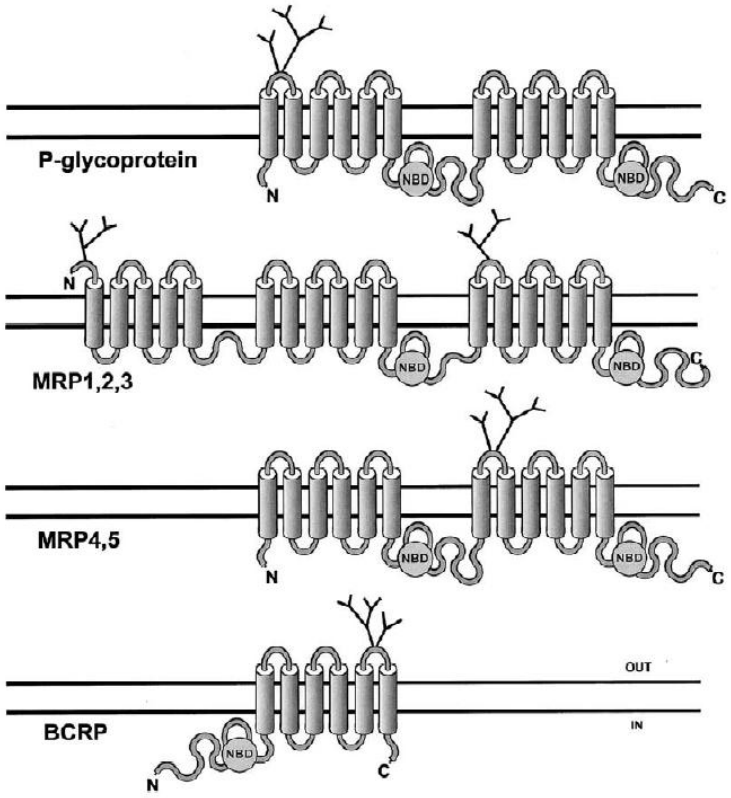
placentární bariéru. Studium aktivní složky ochranné placentární bariéry bylo hlavním cílem této disertační práce, proto budou tyto mechanismy podrobněji popsány v následujících kapitolách.

2.1. Efluxní ABC transportéry

Efluxní ABC (ATP Binding Cassette) transportéry patří mezi membránové transportní proteiny, které aktivně, za spotřeby ATP, "pumpují" substráty směrem ven z buňky proti koncentračnímu gradientu (eflux). Do dnešní doby bylo u člověka popsáno a klasifikováno 49 ABC transportérů. Vyskytují se v řadě tkání (mozek, varlata, placenta, játra, střevní epitel, ledviny apod.) (Langmann et al., 2003), kde modulací absorpce, distribuce a exkrece široké škály látek (produkty metabolismu, toxiny, léčiva, endogenní lipidy, nukleotidy, steroidní látky) plní především ochrannou a detoxikační funkci (Bodo et al., 2003; Leslie et al., 2005). U některých z těchto transportérů byla navíc popsána souvislost s fenoménem tzv. mnohočetné lékové rezistence především vůči cytotoxickým látkám užívaným v léčbě rakoviny (Nooter and Stoter, 1996; Kuo, 2007; Sharom, 2008).

Základní struktura efluxních transportérů je vždy stejná. Tvoří ji různý počet **transmembránových domén (TMD)**, z nichž každá se skládá z šesti transmembránových segmentů, a **nukleotidy vázajících domén (NBD)**, nacházejících se na N- nebo C-konci molekuly proteinu (Obr. 3). Podle počtu domén mohou být efluxní transportéry klasifikovány buď jako „plné“ transportéry, tvořené dvěma až třemi TMD a dvěma NBD, nebo jako „polo-“ transportéry, které tvoří pouze jedna TMD a jedna NBD (Hyde et al., 1990). Transportéry z druhé zmíněné skupiny se skládají v homo- či heterodimery, tak aby byl vytvořen funkční transportér. Geny kódující ABC transportéry jsou podle podobnosti ve struktuře genu, pořadí domén a homologie TMD a NBD klasifikovány do sedmi podrodin (A – G) (Dean and Allikmets, 2001; Dean et al., 2001) (přehledné informace na: http://nutrigene.4t.com/human.abc.htm). Do skupiny ABC transportérů, označovaných jako **lékové efluxní transportéry** se v současné době řadí **P-glykoprotein** (ABCB1, P-gp, MDR1), skupina tzv. **„multidrug rezistance-associated proteins“** (ABCC1-6, ABCC10-12; MRP 1-9) a **„breast cancer resistance protein“** (ABCG2, BCRP) (Obr. 3). V současné době se předpokládá, že

nejdůležitějšími efluxními transportéry placentární bariéry jsou P-gp a BCRP, tyto transportéry budou proto podrobněji popsány v následujícím textu.



Obr 3. *Základní struktura ABC transportérů tvořená různým počtem TMD a NBD. Převzato z přehledové publikace autorů Schinkel a Jonker (Schinkel and Jonker, 2003).*

2.1.1. P-glykoprotein

P-glykoprotein (P-gp) je prvním objeveným lékovým transportérem a do dnešní doby i nejlépe prostudovaným. U člověka se vyskytují dvě varianty P-gp: *MDR1* a *MDR3*, u hlodavců jsou to varianty tři: *mdr1a*, *mdr1b* a *mdr2*, přičemž pouze geny *MDR1*, *mdr1a* a *mdr1b* kódují protein, který se chová jako lékový efluxní transportér. Molekula P-gp o velikosti 170 kDa se skládá ze dvou TMD, každou z nich tvoří šest transmembránových segmentů a jedna NBD (Obr. 3). Charakteristickou vlastností P-gp je schopnost transportovat širokou škálu chemicky a strukturně odlišných látek. Substráty P-gp jsou především neutrální či mírně bazické látky o molekulové hmotnosti 200 - 1800 Da (Tab. 1). Ačkoliv byly popsány

i endogenní substráty P-gp (Huang et al., 1998; King et al., 2001; Raggers et al., 2001), předpokládá se, že hlavní fyziologickou funkcí tohoto transportéru je ochrana buněk, tkání a orgánů před potenciálně škodlivými látkami z vnějšího prostředí (Ganapathy et al., 2000).

Dosavadní poznatky o expresi, lokalizaci a funkci P-gp v placentě jsou přehledně shrnuty v článku M. Čečkové a kol. (Ceckova-Novotna et al., 2006). V placentě člověka byl P-gp detekován ve vrstvě syncytiotrofoblastu na úrovni mRNA i proteinu (Cordon-Cardo et al., 1989; Bremer et al., 1992), zatímco v endotelu kapilár choriových klků nebyla přítomnost P-gp potvrzena. Stejně tak, byla exprese P-gp prokázána i v placentě potkana (Leazer and Klaassen, 2003; Pavek et al., 2003). Imunohistochemické studie prokázaly lokalizaci P-gp výhradně na apikální membráně kartáčového lemu syncytiotrofoblastu (MacFarland et al., 1994). Tato lokalizace je příhodná pro plnění ochranné funkce P-gp, která vyplývá z aktivního transportu cizorodých látek směrem ven z plodu. Aktivita placentárního P-gp byla poprvé ověřena na membránových vezikulech připravených z trofoblastu lidské placenty (Nakamura et al., 1997). Funkční aktivita P-gp v placentě byla následně prokázána také v primární kultuře lidského trofoblastu a BeWo buněčné linii odvozené z choriokarcinomu placenty v *in-vitro* studiích využívajících substráty a inhibitory P-gp (Ushigome et al., 2000; Utoguchi et al., 2000). Na polarizovaných BeWo buňkách rostoucích v monovrstvě bylo navíc jasně demonstrováno, že transport digoxinu, vinblastinu a vinkristinu (substrátů P-gp) je výraznější ve směru z bazolaterální strany na stranu apikální (Ushigome et al., 2000).

Rozdílná distribuce xenobiotik na obou stranách placentární bariéry způsobená aktivitou P-gp byla popsána také ve studiích s perfundovanou lidskou placentou *in-vitro* (Sudhakaran et al., 2005; Nekhayeva et al., 2006; Nanovskaya et al., 2008). Na zvířecích modelech geneticky modifikovaných mdr1a/b deficientních myší, postrádajících funkční P-gp, byl jednoznačně demonstrován význam tohoto transportéru v ochraně plodu před potenciálně toxickými a teratogenními látkami *in-vivo* (Lankas et al., 1998; Smit et al., 1999). Konečně, s využitím techniky duální perfúze placenty potkana *in-situ* byl přímo prokázán vliv aktivity placentárního P-gp na farmakokinetiku cyklosporinu (Pavek et al., 2001) a rhodaminu 123 (Pavek et al., 2003).

2.1.2. BCRP

Na rozdíl od P-gp, byl BCRP objeven relativně nedávno. Poprvé byl detekován v buněčné linii MCF-7 jako transportér způsobující rezistenci buněk vůči mitoxantronu a daunorubicinu (Doyle et al., 1998), odtud byl odvozen i jeho název – „breast cancer resistance protein". BCRP transportér je kódován pouze jedním genem jak u člověka, tak u hlodavců (*ABCG2*/*abcg2*). Molekulu proteinu o velikosti cca 75 kDa tvoří pouze jedna TMD a jedna NBD, BCRP je tak typický „polo"-transportér. Podobně jako P-gp je BCRP schopný přenášet širokou škálu strukturně různorodých látek. Pozoruhodný je částečný překryv v substrátové specifitě s P-gp (Tab. 1), nicméně oproti P-gp je BCRP schopen transportovat také kladně i záporně nabité molekuly, organické anionty a sulfátové konjugáty.

Vysoká exprese mRNA BCRP v nenádorové tkáni byla poprvé detekována právě v placentě člověka (Allikmets et al., 1998) a později i v řadě dalších tkání (Maliepaard et al., 2001). Výsledky prací zabývajících se změnami exprese BCRP v průběhu zrání placenty se značně rozchází, zdá se ale, že nejvyšší hladiny BCRP v placentě jsou ve střední fázi gravidity a ke konci pozvolna klesají (Yasuda et al., 2005; Meyer zu Schwabedissen et al., 2006; Cygalova et al., 2008). V placentě je BCRP lokalizován na apikální membráně trofoblastu a jeho úloha pravděpodobně spočívá, podobně jako u P-gp, v omezení průchodu cizorodých látek přes placentární bariéru. Kromě toho je v poslední době diskutována i fyziologická role BCRP v regulaci syntézy estrogenů v placentě (Grube et al., 2007) a ochraně trofoblastu před apoptózou (Evseenko et al., 2007a; Evseenko et al., 2007b).

Aktivita placentárního BCRP byla studována jak na úrovni *in-vitro* tak *in-vivo*. V membránových vezikulách izolovaných z lidské placenty byla potvrzena transportní aktivita BCRP s využitím mitoxantronu a glyburidu a inhibitoru BCRP - novobiocinu (Kolwankar et al., 2005; Gedeon et al., 2008). Funkční aktivita BCRP byla potvrzena také s využitím primární kultury placentárního trofoblastu (Evseenko et al., 2006) a BeWo buněčné linie (Ceckova et al., 2006). Ve studiích sledujících vliv BCRP na farmakokinetiku průchodu substrátů BCRP nitrofurantoinu a glyburidu přes placentu byl s úspěchem využit *in-vivo* model Bcrp1 deficientních myší (Zhang et al., 2007; Zhou et al., 2008) a *in-vitro* duální perfúze lidské placenty (Kraemer et al., 2006). V naší laboratoři jsme prokázali vliv BCRP na

farmakokinetiku modelových substrátů *in-situ* s využitím duálně perfundované placenty potkana (Staud et al., 2006b) – podrobněji v oddílu IV. této práce.

Tabulka 1 *Vybrané substráty a inhibitory transportérů P-gp a BCRP.*

P-glykoprotein		**BCRP**	
Substráty	***Inhibitory***	***Substráty***	***Inhibitory***
Cytostatika vinblastin, vinkristin paklitaxel, docetaxel doxorubicin, daunorubicin, epirubicin bisantren, mitoxantron etoposid, teniposid aktinomycin D metotrexat topotekan **HIV proteázové inhibitory** sakvinavir, ritonavir, nelfinavir, indinavir, lopinavir, amprenavir **Imunosupresiva** cyklosporin A, FK506 **Antibiotika** erytromycin, valinomycin, gramicidin B, tetracyklin, ofloxacin **H_2-antihistaminika** cimetidin **Antiemetika** domperidon, ondansetron **Antiarytmika** digoxin, verapamil **Antiepileptika** fenytoin **Kortikoidy** dexametazon, hydrokortizon, kortikosteron, triamcinolon **Jiné** Rhodamin 123 Hoechst 33342	valspodar (PSC 833) GF120918 LY-335979 MS-209 R-101933 tariquidar (XR-9576) mitotan (NSC-38721)	**Cytostatika** mitoxantron, bisantren, BBR-390 metotrexát topotekan, irinotekan, diflomotekan, SN-38 azidothymidin NB-506, J-107088 CI1033 imatinib flavopyridol **Antivirotika** lamivudin **H_2-antihistaminika** cimetidin **Endogenní substráty** estron-3-sulfát, 17β-estradiolsulfát, 17β-estradiol 17-(β-glu kuronid) kyselina listová protoporfyrin IX (PPIX) **Jiné** BODIPY-prazosin Hoechst 33342	GF120918 Fumitremorgin C a jeho analoga gefitinib (Iressa, ZD1839) imatinib (STI571, Gleevec) biricodar (VX-710) EKI-785 CI1033 UCN-01 novobiocin estron diethylstilbestrol tamoxifen a deriváty (TAG-11, TAG0139) reserpin tryprostatin A flavonoidy (chrysin, biochanin) ritonavir saquinavir nelfinavir omeprazol taxany (ortataxel, tRA96023)

** Zpracováno a upraveno dle (Schinkel and Jonker, 2003; Hoffmann and Kroemer, 2004; Mao and Unadkat, 2005; Staud and Pavek, 2005).*

2.2. Biotransformační enzymy

Během intrauteriního života probíhá v placentě bohatá enzymatická aktivita, která se podílí na ochraně plodu před zvýšenou expozicí vůči rizikovým látkám přítomným v mateřské krvi. Ačkoliv placentární metabolický systém a substrátové spektrum jsou v porovnání s jaterní tkání výrazně redukované (tvoří cca 10% jaterní aktivity), v placentě byla prokázána přítomnost řady enzymů I. a II. fáze metabolismu. Některé enzymy katalyzují metabolismus endogenních látek, jiné rozeznávají jako substráty i pestrou škálu xenobiotik. Tyto enzymy tvoří spolu s transportními proteiny aktivní složku ochranné placentární bariéry (Marin et al., 2004; Syme et al., 2004).

Enzymy cytochromu P450 (CYP450) jsou součástí komplexního enzymatického systému, který zajišťuje oxidativní metabolismus endogenních i exogenních látek. Funkčně mohou být enzymy CYP450 rozděleny do dvou skupin: a) enzymy CYP5 - 27 se uplatňují v syntéze endogenních molekul, b) enzymy z rodiny CYP1 - 4 jsou zodpovědné za metabolismus xenobiotik. V placentě tvoří převážnou většinu enzymů CYP450 enzymy katalyzující metabolismus endogenních steroidních molekul (především aromatáza - CYP19). Xenobiotika-metabolizující CYP450 jsou zde zastoupeny minoritně především enzymy: CYP1A1, CYP2E1, CYP2F1, CYP3A4, CYP3A5, CYP3A7 a CYP4B1 (Hakkola et al., 1996a; Hakkola et al., 1996b). Vedle CYP450 byla v placentě popsána exprese a aktivita řady enzymů II. fáze metabolismu, které katalyzují konjugační reakce: glukuronyltransferáza, N-acetyltransferáza, glutathion-S-transferáza, sulfotransferáza a epoxid hydroláza (Syme et al., 2004). Do jaké míry mají tyto enzymy význam v ochranné funkci placenty však dodnes není zcela jasné.

Významnou složkou aktivní placentární bariéry je enzym 11β-hydroxysteroid dehydrogenáza (11β-HSD) zajišťující biotransformaci endogenních steroidů, především glukokortikoidů (GK). Je dobře známo, že GK mají zásadní vliv na dozrávání orgánů plodu, růst a načasování doby porodu. Na druhou stranu, vysoké hladiny GK v krvi plodu mohou způsobit růstovou retardaci (intra-uterine growth retardation) a následné zvýšené riziko výskytu metabolických a kardiovaskulárních chorob v dospělosti. V této souvislosti se dokonce často hovoří o jevu tzv. "**prenatálního programování**" (viz. přehledové články (Seckl, 2004; Seckl and Holmes, 2007)). Pro normální průběh těhotenství a zdravý vývoj jedince je proto

nezbytnou nutností velmi přesná regulace hladin GK v krvi plodu. Z tohoto pohledu je placentární 11β-HSD považována za klíčový enzym, který reguluje průchod GK přes placentu a tím zajišťuje udržení optimálních koncentrací GK, kterým je vystaven vyvíjející se plod.

2.2.1. 11β-hydroxysteroid dehydrogenáza

11β-HSD katalyzuje oxidačně-redukční děje spojené s přeměnou metabolicky aktivních steroidů (kortisol, kortiksteron) a jejich neaktivních keto-derivátů (kortison, 11-dehydrokortikosteron). V současné době jsou známy dva enzymy, produkty dvou různých genů, které mají dehydrogenázovou aktivitu. 11β-HSD typu 1 (11β-HSD1) je $NADP^+/H$ dependentní enzym s K_m v mikromolární oblasti. Ačkoliv 11β-HSD1 je schopna katalyzovat oxidačně-redukční reakci v obou směrech, působí primárně jako reduktáza, tzn. katalyzuje tvorbu aktivních forem GK. Oproti tomu, 11β-HSD typu 2 (11β-HSD2) je NAD^+ dependentní vysokoafinitní enzym (K_m v nanomolární oblasti), který působí výhradně jako dehydrogenáza a je zodpovědný za inaktivaci aktivních forem GK. V placentě byla popsána dvojí role 11β-HSD (Burton and Waddell, 1999). Za hlavní funkci se považuje vytváření aktivní bariéry proti endogenním GK a udržování optimálních hladin GK ve fetální krvi zajišťujících normální vývoj plodu. Kromě toho, 11β-HSD významně moduluje účinky steroidních hormonů přes glukokortikoidní a mineralokortikoidní receptor. Tím ovlivňuje řadu buněčných funkcí v placentě, od diferenciace trofoblasu až po syntézu peptidových a steroidních hormonů.

Z hlediska ochranné funkce se předpokládá, že dominantní formou placentární bariéry je 11β-HSD2 se svojí dehydrogenázovou aktivitou. Tento předpoklad potvrzují i studie zabývající se expresí a aktivitou obou typů 11β-HSD v placentě. Zatímco přítomnost 11β-HSD2 byla potvrzena v placentě člověka i potkana na úrovni mRNA i proteinu a v placentární bariéře lokalizována ve vrstvě syncytiotrofoblastu, exprese 11β-HSD1 byla v placentě člověka detekována pouze v endoteliálních buňkách a extravilózním trofoblastu (Sun et al., 1997; Driver et al., 2001; Staud et al., 2006a). Na druhou stranu, u jiných živočišných druhů, např. u paviána či potkana, byla potvrzena přítomnost 11β-HSD1 také v placentárním syncytiotrofoblastu (Burton et al., 1996; Pepe et al., 1996). Ve studiích sledujících

oxidačně-redukční aktivitu 11β-HSD v placentách člověka i potkana byla pozorována převažující dehydrogenázová aktivita 11β-HSD2, která se navíc v průběhu dozrávání placenty zvyšovala (Stewart and Krozowski, 1999). Nicméně, výsledky těchto studií může částečně zkreslovat fakt, že oxo-reduktázová aktivita 11β-HSD1 v placentě je jen obtížně detekovatelná díky její relativní nestabilitě (Burton and Waddell, 1999). Fyziologický význam 11β-HSD1 v metabolismu GK proto nelze zcela vyloučit.

Ze současných poznatků vyplývá, že exprese, potažmo aktivita, obou typů placentární 11β-HSD podléhá významným druhově-specifickým rozdílům. Navíc, podobně jako u řady jiných enzymů, není exprese a aktivita 11β-HSD v placentě v průběhu gravidity stabilní, což je pravděpodobně důsledek tkáňově specifické regulace nejen endogenními, ale i řadou exogenních látek, včetně léčiv (Hardy et al., 1999; Tremblay et al., 1999; Lanz et al., 2003; Suzuki et al., 2003; Atanasov et al., 2005). S ohledem na zásadní význam, který představuje placentární glukokortikoidní bariéra pro vývoj jedince, je nezbytné poznat a popsat vliv těchto látek na expresi a funkční aktivitu placentární 11β-HSD.

3. Metody studia placentární bariéry

Těhotné ženy jsou denně vystavovány působení rostoucího množství cizorodých látek pocházejících z ovzduší, jídla, pití, léků apod. Účinky těchto látek na průběh těhotenství a vývoj plodu nejsou v převážné většině případů známy, stejně jako kinetika jejich průchodu přes placentární bariéru. Z etických důvodů nelze provádět studium rizik spojených s působením cizorodých látek přímo na těhotných ženách. Omezené je také zařazování gravidních žen do klinických studií vyhodnocujících účinky jednotlivých léčivých látek. Většina informací je proto získávána ze studií prováděných na zvířecích modelech a/nebo s využitím alternativních technik a modelů placentární bariéry, které umožňují sledovat transport a metabolismus látek v placentě za fyziologických či experimentálních podmínek (viz. podrobné přehledové články (Bourget et al., 1995; Sastry, 1999; Vahakangas and Myllynen, 2006)). Tyto studie jsou prováděny buď v podmínkách *in-vivo*, *in-vitro* nebo *in-situ*.

3.1. Metody in-vivo

Řada *in-vivo* technik využívá pro studium placentární bariéry březí experimentální zvířata. Na těchto modelech se sledují potenciální teratogenní účinky testovaných látek, jejich vliv na fertilitu, růst, vznik malformací a celkové přežití plodu. Analýzou koncentrací testované látky v krvi a v jednotlivých orgánech lze získat informace o distribuci látky v těle matky či plodu. S využitím radioaktivně značených látek lze navíc sledovat distribuci pomocí autoradiografie. Pro objasnění fyziologických funkcí vybraných transportérů či metabolických enzymů v placentě se také využívají geneticky manipulovaná experimentální zvířata, nejčastěji myši či potkani, postrádající gen kódující vybraný protein. Na těchto modelech lze pak v porovnání se zvířaty standardního fenotypu sledovat rozdíly v průběhu gravidity a vývoji plodu.

U lidí je možné odhadovat míru průchodu sledované molekuly do krve plodu na základě porovnání koncentrací v krvi matky a v pupečníkové krvi odebírané těsně po porodu. Další informace o rychlosti a směru transportu molekul mohou být získány selektivním odběrem a analýzou krve z pupečníkové arterie a žíly. Tento způsob však neposkytuje komplexní informace, které by zohledňovaly změny průchodu látek přes placentu např. v průběhu těhotenství, při různých patologických stavech nebo vlivem jiné medikace.

3.2. Metody in-vitro

Široce rozšířeným modelem materno-fetální bariéry jsou *in-vitro* buněčné kultury odvozené z placentárního trofoblastu lidského nebo zvířecího původu. Ačkoliv data získaná ze studií prováděných v podmínkách *in-vitro* nereflektují plně fyziologické a biochemické změny probíhající v placentě, poskytují cenné informace zejména při provádění pilotních experimentů s novými typy molekul. Navíc mohou částečně nahradit zvířecí modely, čímž významně omezují počty experimentálních zvířat v jednotlivých studiích. Pro *in-vitro* experimenty se využívají jak primární kultury placentárního trofoblastu tak nádorové buněčné linie, například BeWo, Jeg-3, JAr, HRP-1 apod. Tyto buňky mají podobnou expresi transportních proteinů a metabolických enzymů jako původní trofoblast a jsou proto vhodné pro sledování

transportu a biotransformace látek v placentární bariéře. Pro tyto účely lze vytvořit jednoduchý model placentární bariéry kultivací buněk v polarizované monovrstvě na speciálních membránách.

Transportní mechanismy uplatňující se v placentě lze v *in-vitro* podmínkách sledovat také s pomocí membránových vezikul izolovaných z placentárního trofoblastu. Ke studiu enzymatické aktivity a metabolismu látek v placentě se vyžívají placentární mikrosomy, připravené diferenciální centrifugací placenty. Výhodou mikrosomů je možnost sledovat metabolickou aktivitu v placentách jak v časných tak v pozdních stádiích těhotenství.

3.3. Metody in-situ

Relativně náročná, avšak velmi cenná je metoda duální perfúze lidské či zvířecí placenty, vhodná pro přímé studium kinetiky průchodu látek přes placentární bariéru. Tato technika umožňuje sledovat transport vybraných molekul v materno-fetálním směru a také odhadnout velikost placentární clearance dané látky již přítomné v cirkulaci plodu. S využitím specifických substrátů či inhibitorů lze na tomto modelu studovat význam jednotlivých transportních proteinů nebo metabolických enzymů v placentární bariéře a ochraně plodu před cizorodými látkami.

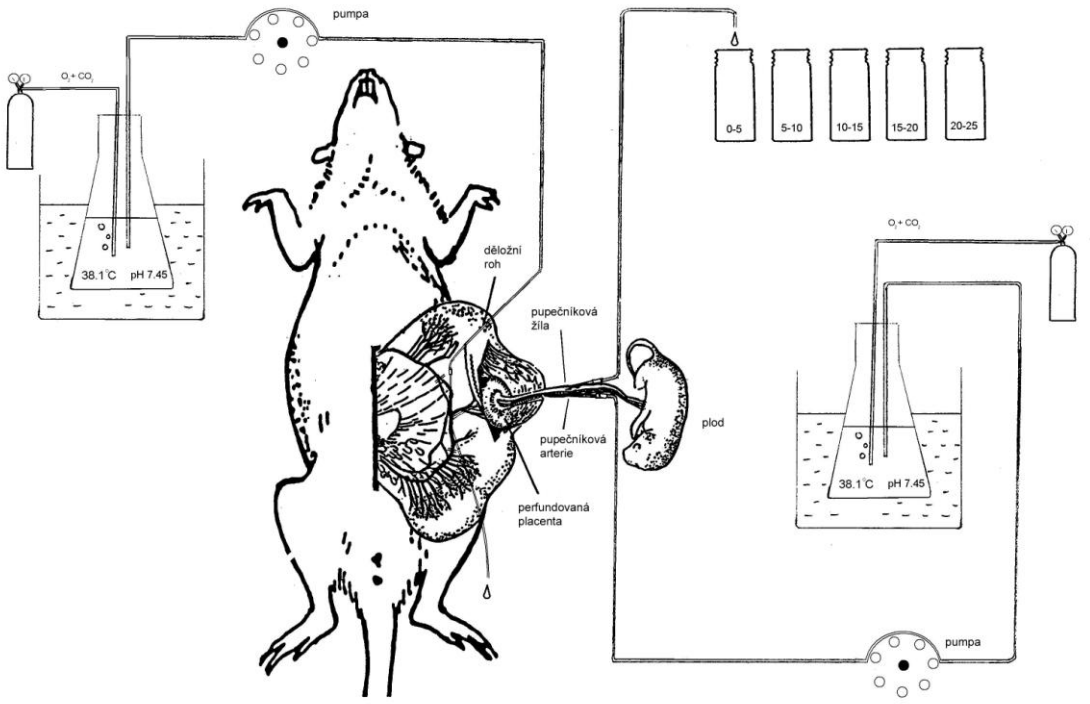
Extrapolace dat získaných z perfúzí celých placent není vždy jednoznačná. Aktivita enzymů a transportních proteinů v lidské placentě na konci těhotenství nemusí odpovídat stavu v prvním či druhém trimestru. Navíc, během porodu dochází k poškození tkáně, které může ovlivnit funkčnost celého systému. Při perfúzích zvířecích placent je zase nutné brát v úvahu mezidruhové odlišnosti ve struktuře placentární bariéry. Na druhou stranu, nespornou výhodou zvířecích modelů je možnost sledovat změny v transportní a metabolické aktivitě placentární bariéry způsobené například prenatální terapií vybranými lékovými induktory či inhibitory, což u člověka není z etických důvodů možné.

V této disertační práci byla ke studiu kinetiky transportu a metabolismu vybraných molekul v placentě využívána metoda duální perfúze placenty potkana. Tato technika bude proto podrobněji popsána v následující kapitole.

3.4. Duální perfúze placenty potkana

Metoda duální perfúze placenty potkana je cenná technika umožňující studovat v podmínkách *in-situ* aktivitu jednotlivých složek placentární bariéry a jejich vliv na kinetiku transportu různorodých látek přes placentu. Průchod substrátů transportních proteinů či metabolických enzymů přes placentu lze sledovat jak ve směru z matky do plodu (materno-fetální) tak ve směru opačném (feto-maternální).

Ve všech studiích této práce byla duální perfúze prováděna u zvířat v 21. dnu březosti. Jednotlivé experimenty probíhaly ve dvojím možném uspořádání. Pro studium farmakokinetiky průchodu modelových substrátů přes placentu bylo použito **otevřené uspořádání** (Obr. 4), ve kterém byly obě strany placenty (mateřská a fetální) perfundovány bez recirkulace. Sledovaný substrát byl v tomto případě přidán do rezervoáru na mateřské (materno-fetální transport) či plodové (feto-maternální transport) straně a analyzován v daných časových intervalech ve vzorcích perfuzátu vytékajícího z pupečníkové cévy na straně plodu. Pro studium schopnosti BCRP transportéru eliminovat substrát z krve plodu proti koncentračnímu gradientu byl použit **polouzavřený systém**. V tomto uspořádání byl do mateřského i plodového zásobníku přidán substrát o stejné koncentraci, přičemž fetální perfuzát byl po určitou dobu recirkulován. Změny v koncentraci substrátu byly analyzovány ve vzorcích sbíraných v daných časových intervalech z obou zásobníků.



Obr 4. *Schématické uspořádání duální perfúze placenty potkana v otevřeném uspořádání bez recirkulace.*

3.4.1. Farmakokinetická analýza aktivity efluxních transportérů v placentě

Pro matematický popis farmakokinetiky průchodu modelových substrátů transportních proteinů jsme použili koncept placentární clearance. Pro kalkulaci celkové materno-fetální (Cl_{Tmf}) a feto-maternání (Cl_{Tfm}) clearance normalizované k hmotnosti placenty byly použity následující matematické vztahy:

$$Cl_{Tmf} = \frac{C_{fv} \cdot Q_f}{C_{ma} \cdot w_p}$$

$$ER = \frac{C_{fa} \cdot C_{fv}}{C_{fa}}$$

$$Cl_{Tfm} = \frac{ER \cdot Q_r}{w_p}$$

kde C_{fv} je koncentrace substrátu v perfuzátu sbíraném z pupečníkové žíly, Q_f rychlost průtoku pupečníkovou arterií, C_{ma} koncentrace substrátu v zásobníku na mateřské straně, w_p váha placenty, *ER* extrakční poměr znázorňující schopnost placenty odstraňovat substrát z cirkulace plodu a C_{fa} koncentrace substrátu v zásobníku na plodové straně.

Pro rozlišení mezi příspěvkem pasivní difúze a aktivního transportu na celkovou placentární clearance jsme vycházeli z úvahy, že celkový průchod modelového substrátu přes placentu je funkcí pasivní difúze a aktivního transportu. Potom lze vyjádřit celkovou placentární clearance následujícími vztahy:

$$Cl_{Tmf} = Cl_{pd} - Cl_{efflux}$$

$$Cl_{Tfm} = Cl_{pd} + Cl_{efflux}$$

kde Cl_{pd} je clearance pasivní difúze a Cl_{efflux} vyjadřuje aktivitu efluxního transportéru.

Protože aktivní transport prostřednictvím efluxního transportéru je nelineární proces, po dosažení hraniční koncentrace substrátu dojde k vysycení transportéru a celková clearance se pak bude blížit clearance pasivní difúze. Kinetiku tohoto nelineárního děje lze popsat Michaelis-Mentenovou rovnicí. Po jejím dosazení do výše uvedených vztahů jsme získali rovnice pro vyjádření hodnot clearance v závislosti na koncentraci substrátu:

$$Cl_{Tmf} = Cl_{pd} - \frac{V_{max}}{K_m + C_{ma}}$$

$$Cl_{Tfm} = Cl_{pd} + \frac{V_{max}}{K_m + C_{fa}}$$

kde V_{max} je maximální rychlost transportu dané látky, K_m koncentrace substrátu při které je dosaženo poloviny maximální rychlosti transportu a $C_{ma(fa)}$ je koncentrace v mateřské či plodové krvi.

3.4.2. Analýza aktivity 11β-hydroxysteroid dehydrogenázy v placentě

Při hodnocení metabolické aktivity 11β-hydroxysteroid dehydrogenázy typu 2 (11β-HSD2) v placentě potkana byl jako modelový substrát použit kortikosteron. Průchod kortikosteronu přes placentu byl sledován v materno-fetálním směru, jeho metabolismus v placentě pak vyhodnocován na základě analýzy kortikosteronu a jeho metabolitů v perfuzátu sbíraném na fetální straně v daných časových intervalech. Z naměřených hodnot pak byla kalkulována konverzní kapacita enzymu 11β-HSD2 (EC_{hsd}) podle následujícího vztahu:

$$EC_{hsd} = \frac{C_m}{C_s + C_m}$$

kde C_m je koncentrace metabolitu (11-dehydrokortikosteron) a C_s koncentrace substrátu (kortikosteron) ve vzorcích perfuzátu.

4. Cíle předkládané disertační práce:

Hlavním cílem předkládané práce bylo studium role efluxních transportérů a biotransformačních enzymů v detoxikační funkci placenty. Jmenovitě:

1. Studium exprese a transportní aktivity BCRP v buněčné linii HRP-1 a jeho vlivu na intracelulární kumulaci farmak.

2. Studium exprese a transportní aktivity BCRP v placentě potkana, především vlivu BCRP na farmakokinetiku průchodu léčiv přes placentu.

3. Studium vlivu 11β-HSD na hladiny GK v krvi plodu v průběhu březosti potkana.

4. Sledování vlivu syntetických GK podávaných matce na konci březosti na funkční expresi 11β-HSD v placentě potkana.

5. Podíl práce doktorandky v předkládaných publikacích

U kapitoly IV. je předkladatelka této disertační práce první autorkou, v případě kapitol II. a III. spoluautorkou.

V práci uvedené v části II. se autorka podílela na provádění experimentů s duální perfúzí placenty potkana, odběru vzorků, jejich zpracování a analýze. Pomocí zavedené metody real-time RT-PCR analyzovala expresi genu pro BCRP v placentě potkana a placentární buněčné linii HRP-1.
Ve studii III. zavedla autorka metodu pro studium průchodu kortikosteronu přes placentární bariéru *in-vivo* v různých stadiích březosti.
Ve studii uvedené v části IV. této práce koordinovala provádění funkčních experimentů, prováděla odběr vzorků placent, jejich zpracování a následnou analýzu exprese 11β-HSD na úrovni mRNA metodou real-time RT-PCR.

Autorka disertace sepsala rukopis práce uvedené v kapitole IV. Ve studii II., kde je uvedena jako spoluautorka, se podílela na sepsání částí týkajících se metody real-time RT-PCR a v práci III. sepsala metodickou část *in-vivo* studia průchodu kortikosteronu přes placentu potkana.

Autorem systémů pro real-time RT-PCR detekci a kvantifikaci genové exprese je PharmDr. Antonín Libra, PhD. (Generi Biotech s.r.o.)
Detekci proteinu metodou Western blot a imunohistochemickou lokalizaci BCRP v placentě potkana prováděli PharmDr. Martina Čečková, PhD. z Katedry farmakologie a toxikologie a Mgr. Kateřina Pospěchová z Katedry lékařských věd Farmaceutické fakulty.
Expresi 11β-HSD na úrovni proteinu metodou Western blot prováděla RNDr. Karla Vágnerová, PhD. z pracoviště Fyziologického ústavu AV ČR vedeného Prof. RNDr. Jiřím Páchou, DrSc. Analýzu kortikosteronu metodou HPLC ve vzorcích perfuzátů prováděl Ing. Ivan Mikšík, PhD. z Fyziologického ústavu AV ČR.

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II.

EXPRESSION AND TRANSPORT ACTIVITY OF BREAST CANCER RESISTANCE PROTEIN (Bcrp/Abcg2) IN DUALLY PERFUSED RAT PLACENTA AND HRP-1 CELL LINE

0022-3565/06/3191-53–62$20.00
THE JOURNAL OF PHARMACOLOGY AND EXPERIMENTAL THERAPEUTICS
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JPET 319:53–62, 2006

Vol. 319, No. 1
105023/3138313
Printed in U.S.A.

Expression and Transport Activity of Breast Cancer Resistance Protein (Bcrp/Abcg2) in Dually Perfused Rat Placenta and HRP-1 Cell Line

Frantisek Staud, Zuzana Vackova, Katerina Pospechova, Petr Pavek, Martina Ceckova, Antonin Libra, Lenka Cygalova, Petr Nachtigal, and Zdenek Fendrich

Departments of Pharmacology and Toxicology (F.S., Z.V., P.P., M.C., A.L., L.C., Z.F.) and Biomedical Sciences (K.P., P.N.), Faculty of Pharmacy in Hradec Kralove, Charles University, Prague, Czech Republic

Received March 22, 2006; accepted June 28, 2006

ABSTRACT

Breast cancer resistance protein (BCRP/ABCG2) is a member of the ATP-binding cassette transporter family that recognizes a variety of chemically unrelated compounds. Its expression has been revealed in many mammal tissues, including placenta. The purpose of this study was to describe its role in transplacental pharmacokinetics using rat placental HRP-1 cell line and dually perfused rat placenta. In HRP-1 cells, expression of Bcrp, but not P-glycoprotein, was revealed at mRNA and protein levels. Cell accumulation studies confirmed Bcrp-dependent uptake of BODIPY FL prazosin. In the placental perfusion studies, a pharmacokinetic model was applied to distinguish between passive and Bcrp-mediated transplacental passage of cimetidine as a model substrate. Bcrp was shown to act in a concentration-dependent manner and to hinder maternal-to-fetal transport of the drug. Fetal-to-maternal clearance of cimetidine was found to be 25 times higher than that in the opposite direction; this asymmetry was partly eliminated by BCRP inhibitors fumitremorgin C (2 μM) or *N*-(4-[2-(1,2,3,4-tetrahydro-6,7-dimethoxy-2-isoquinolinyl)ethyl]-phenyl)-9,10-dihydro-5-methoxy-9-oxo-4-acridine carboxamide (GF120918; 2 μM) and abolished at high cimetidine concentrations (1000 μM). When fetal perfusate was recirculated, Bcrp was found to actively remove cimetidine from the fetal compartment to the maternal compartment even against a concentration gradient and to establish a 2-fold maternal-to-fetal concentration ratio. Based on our results, we propose a two-level defensive role of Bcrp in the rat placenta in which the transporter 1) reduces passage of its substrates from mother to fetus but also 2) removes the drug already present in the fetal circulation.

Placenta is an organ that brings maternal and fetal blood circulations into proximity, allowing mutual interchange of nutrients and waste products. Conversely, placenta forms a barrier to protect the fetus against harmful endo-and exogenous compounds from maternal circulation. As a barrier, the human and rodent placenta had for long been supposed to present only a mechanical obstruction formed by fetal endothelia, basal membranes, and syncytiotrophoblast. However, over the past two decades, a variety of metabolizing enzymes and drug efflux transporters of the ATP-binding cassette (ABC) transporter family have been localized in placental trophoblast (Marin et al., 2004; Syme et al., 2004). These proteins are thought to strengthen, in an active and capacity-limited manner, placental barrier role and help in protecting the fetus.

Drug efflux transporters of the ABC family are membrane-embedded proteins that limit intracellular concentration of substrates by pumping them out of cell through an active, energy-dependent mechanism (Schinkel and Jonker, 2003). The most intensively studied drug efflux transporters to date have been P-glycoprotein (P-gp; ABCB1), breast cancer resistance protein (BCRP; ABCG2) and multidrug resistance-associated proteins 1 and 2 (ABCC1 and ABCC2), all of which were found to be responsible for the phenomenon of multidrug resistance in cancer therapy (Fischer et al., 2005). In addition, because of their extensive distribution in nontumorous tissues and wide substrate specificity, these proteins

This work was supported by Grants 151/2004/C/FaF and 104/2005/C from Grant Agency of Charles University, Prague, Czech Republic.
Article, publication date, and citation information can be found at http://jpet.aspetjournals.org.
doi:10.1124/jpet.106.105023.

ABBREVIATIONS: ABC, ATP-binding cassette; P-gp, P-glycoprotein; BCRP/Bcrp, human/rodent breast cancer resistance protein; GF120918, *N*-(4-[2-(1,2,3,4-tetrahydro-6,7-dimethoxy-2-isoquinolinyl)ethyl]-phenyl)-9,10-dihydro-5-methoxy-9-oxo-4-acridine carboxamide; RT-PCR, reverse transcription-polymerase chain reaction; mdr/MDR, multidrug resistance; bp, base pair(s); ANOVA, analysis of variance; Ko143, 3-(6-isobutyl-9-methoxy-1,4-dioxo-1,2,3,4,6,7,12,12*a*-octahydro-pyrazino[1′,2′:1,6]pyrido[3,4-*b*]indol-3-yl)-propionic acid *tert*-butyl ester; PSC833, [3′-keto-Bmt[1]]-[Val[2]]-cyclosporine.

significantly affect body disposition of many clinically used drugs. With respect to expression, regulation, function, and clinical relevance, the best described of placental ABC transporters to date is P-glycoprotein (Ceckova-Novotna et al., 2006).

BCRP is the most recently described member of the ABC transporter superfamily (Doyle et al., 1998). Its expression has been assessed in many tissues and cells, including blood-brain barrier, placenta, intestine, various tumors, and "side population" of stem cells (Staud and Pavek, 2005). Because its tissue distribution and substrate specificity overlap noticeably with that of P-gp, it is generally thought that these transporters share a similar role in protecting pharmacological sanctuaries, such as brain and fetus.

Considerable levels of BCRP/Bcrp expression have been detected in placentas of various species. In humans, placental BCRP expression was found to be approximately 10 times higher than that of P-gp (Ceckova et al., 2006). Given the expression pattern and the broad range of substrates, including exogenous (drugs and toxins) and endogenous (steroid conjugates, and porphyrins) compounds (Staud and Pavek, 2005), it is reasonable to assume that BCRP may be an important component of the placental barrier. Kolwankar et al. (2005) confirmed BCRP function in microvillous membrane vesicles of the human placenta. In addition, we have recently described BCRP expression, localization, and function in an in vitro placental model, BeWo cell line (Ceckova et al., 2006). The only functional in vivo experiments proposing Bcrp activity in the placenta were performed by Jonker et al. (2001, 2002) in transgenic mice. Nevertheless, transport activity of this efflux protein and its role in transplacental pharmacokinetics has not been fully evaluated to date.

Because of technical constraints and ethical issues, direct investigation of placental drug transfer under in vivo conditions in human is not feasible; therefore, several alternative experimental methods have been developed to assess potential risk that drugs in maternal circulation present to the fetus (Sastry, 1999). Among these, cell cultures and perfused placentas of various species are widely used models for mechanistic studies to describe transplacental pharmacokinetics, including transport mechanisms and biotransformation. The dually perfused rat placenta, in particular, is a well established model that has been successfully used to investigate placental physiology and pharmacology (Stulc et al., 1995; Kertschanska et al., 2000). In our previous studies, we used this experimental model to evaluate functional activity of P-gp in the rat placenta (Pavek et al., 2001, 2003).

The aim of the present study was to assess the effect of Bcrp on transplacental passage of its substrates. We investigated Bcrp activity both in vitro, using HRP-1 rat placental cell line, and in situ in dually perfused rat term placenta. Using these techniques, we describe Bcrp as an active component of the rat placental barrier that limits maternal-to-fetal and facilitates fetal-to-maternal transport of its substrates.

Materials and Methods

Reagents and Chemicals. Cimetidine and radiolabeled [*N-methyl*-^{3}H]cimetidine were purchased from Sigma-Aldrich (St. Louis, MO) and from GE Healthcare (Little Chalfont, Buckinghamshire, UK), respectively. BODIPY FL prazosin, a common BCRP and P-gp substrate, was obtained from Invitrogen (Carlsbad, CA). Specific BCRP inhibitors Ko143 and fumitremorgin C were donated by Dr. Alfred Schinkel (The Netherlands Cancer Institute, Amsterdam, The Netherlands) or purchased from Alexis Corporation (Lausanne, Switzerland), respectively. Specific P-gp inhibitors, PSC833 and cyclosporine, were gifts kindly provided by Dr. Andrýsek (Ivax Pharmaceuticals, Opava, Czech Republic). Dual BCRP and P-gp inhibitor GF120918 was from GlaxoSmithKline (Greenford, UK). All other compounds were reagent grade.

Cell Cultures. For in vitro accumulation studies, HRP-1 rat trophoblast cells (Soares et al., 1987), received as a generous gift from Dr. Michael Soares (University of Kansas Medical Center, Kansas City, KS) were used. They were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum, 100 units/ml penicillin, 100 μg/ml streptomycin, 1 mM sodium pyruvate, and 50 μM β-mercaptoethanol. Cells from passages 15 to 25 were used in experiments described herein.

Animals. All experiments were approved by the Ethical Committee of the Faculty of Pharmacy in Hradec Kralove (Charles University in Prague, Czech Republic) and were carried out in accordance with the Guide for the Care and Use of Laboratory Animals (1996) and the European Convention for the protection of vertebrate animals used for experimental and other scientific purposes (Strasbourg, 1986). Pregnant Wistar rats were purchased from Biotest Ltd. (Konárovice, Czech Republic) and were maintained in 12/12-h day/night standard conditions with water and pellets ad libitum. Experiments were performed on day 21 of gestation. Fasted rats were anesthetized with pentobarbital (Nembutal; Abbott Laboratories, Abbott Park, IL) in a dose of 40 mg/kg administered into the tail vein.

RNA Isolation and Reverse Transcription-Polymerase Chain Reaction Analysis. Placentas and kidneys were collected on day 21 of gestation from five rats. Five randomly selected placentas from each animal were dissected free of maternal tissues and fetal membranes. Immediately after dissection, the organs were frozen in liquid nitrogen and stored at −70°C until analysis. RNA isolation and reverse transcription were performed as described previously (Novotna et al., 2004). Sequences of mRNAs for target genes were obtained from National Center for Biotechnology Information data base; primers for *mdr1a*, *mdr1b*, and *bcrp* genes were designed using the Vector NTI Suite software (Informax, Bethesda, MD) and are given in Table 1.

RT-PCR analysis was performed on iCycler iQ (Bio-Rad, Hercules, CA). cDNA was amplified with HotStart *Taq* polymerase under the following conditions: 3 mM $MgCl_2$, 0.2 mM dNTP, 0.03 U/μl poly-

TABLE 1
Sequences and specifications of primers used in RT-PCR

Gene	Accession No.	Sequence 5′→3′	Product Length	Localization
			bp	
mdr1a	AF257746	ctg ctc aag tga aag ggg cta ca (f) agc att tct gta tgg tat ctg caa gc (r)	329	2526–2854
mdr1b	AY082609	cgc ttc taa tgt taa agg ggc tat g (f) agc att tct gta tgg tat ctg caa gc (r)	331	2489–2819
Bcrp	NM181381	cca ctg gaa tgc aaa ata gag (f) cct cat agg tag taa gtc aga cac a (r)	188	1340–1527

merase, SYBR Green I in 1:100,000 dilution, and 0.3 μM of each primer; the temperature profile was 95°C for 14 min; 50 times: 95°C for 15 s, 60°C for 20 s, 72°C for 20 s, and 72°C for 5 min; and melting curve program was 72–95°C. Each sample of cDNA was amplified in duplicates. The PCR products were separated by electrophoresis on 2% agarose gel in the presence of ethidium bromide and visualized under ultraviolet light and compared with low-molecular-weight DNA ladder (25–766 bp) (New England BioLabs, Hertsfordshire, UK).

Western Blot Analysis. Cell membrane fractions of placenta tissues and whole-cell lysates were prepared as described previously (Novotna et al., 2004; Ceckova et al., 2006). Protein contents were determined by BCA Protein Assay detection kit (Pierce Chemical, Rockford, IL). Samples containing 100 μg of cell lysate protein or 40 μg of tissue cell membrane were subjected to electrophoresis on 8% SDS-polyacrylamide gels and subsequently electrotransferred onto Hybond-ECL membrane (GE Healthcare). After blocking in 5% nonfat dry milk blocking buffer, the membranes were probed with mouse monoclonal anti-P-gp antibody C219 (Signet Laboratories, Dedham MA) and rabbit polyclonal anti-ABCG2 antibody M-70 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) (1:500 in 1% blocking buffer overnight at 4°C). Incubation with corresponding secondary horseradish peroxidase-conjugated antibody (anti-rabbit, 1:2000 in 1% blocking buffer; anti-mouse, 1:1000; 60 min at room temperature) was used for recognition of the primary antibodies. Immunoreactive proteins were visualized on FOMA Blue Medical X-ray films (FOMA Bohemia; Hradec Kralove, Czech Republic) by ECL Advance Western blotting detection system (GE Healthcare).

Immunohistochemical Localization of Bcrp in the Rat Term Placenta. Preparation of rat placental tissue was performed as described previously (Pavek et al., 2003). The antigen (Bcrp) was unmasked by heating the specimens in sodium citrate buffer, pH 6.0, two times for 8 min each in a microwave oven at 750 W. Slides were incubated with a polyclonal primary antibody for BCRP (M-70; Santa Cruz Biotechnology, Inc.) diluted 1:10 in bovine serum albumin for 15 to 18 h at 4°C. Subsequently, the slides were developed with the secondary antibody goat anti-rabbit IgG conjugated to peroxidase-labeled polymer (DAKO EnVision, Dako North America Inc., Carpinteria, CA) for 30 min. The reaction was visualized using diaminobenzidine (diaminobenzidine substrate-chromogen solution; Dako North America Inc.), and the sections were counterstained by hematoxylin. Slides were examined using computer image analysis (Nikon Eclipse E200 light microscope; Nikon, Tokyo, Japan; PixeLINK PL-A642 digital firewire camera; Vitana Corp., Ottawa, ON, Canada; and LUCIA software, version 4.82; Laboratory Imaging, Prague, Czech Republic). Specificity of the immunostaining was assessed by staining with nonimmune isotype-matched immunoglobulins.

Cellular Uptake Experiments. HRP-1 cells were seeded on 24-well culture plates (1×10^5/well) 2 days before the experiments. Cell culture medium was removed, and cells were washed twice with 500 μl of prewarmed PBS. Cells were then preincubated in Opti-MEM medium with or without inhibitor (1 μM GF120918, 1 μM Ko143, 25 μM verapamil, or 1 μM PSC833) at 37°C in 5% CO_2 for 60 min before fluorescent substrate BODIPY FL prazosin (500 nM) was added. Accumulation was allowed for 2 h at 37°C and was arrested by prompt cooling on ice and removal of medium. Cells were washed with ice-cold PBS and lysed in 1% SDS, and fluorescence was measured after 24 h (Genios Plus; Tecan, Salzburg, Austria). Fluorescence of each well was related to protein content as assessed by BCA Protein Assay detection kit.

Dual Perfusion of the Rat Placenta. The method of dually perfused rat placenta was used as described previously (Pavek et al., 2003). In brief, one uterine horn was excised and submerged in heated Ringer's saline. A catheter was inserted into the uterine artery proximal to the blood vessel supplying a selected placenta and connected with the peristaltic pump. Krebs' perfusion liquid containing 1% albumin was brought from the maternal reservoir at a rate of 1 ml/min. The uterine vein, including the anastomoses to other fetuses, was ligated behind the perfused placenta and cut so that maternal solution could leave the perfused placenta. The selected fetus was separated from the neighboring fetuses by ligatures. The umbilical artery was catheterized using 24-gauge catheter connected to the fetal reservoir and perfused at a rate of 0.5 ml/min. The umbilical vein was catheterized in a similar manner, and the selected fetus was removed. Before the start of each experiment, the fetal vein effluent was collected into preweighed glass vials to check a possible leakage of perfusion solutions from the placenta. In the case of leakage, the experiment was terminated. Maternal and fetal perfusion pressures were maintained at levels close to physiological values and monitored continuously throughout the perfusion experiments as described previously (Pavek et al., 2001). At the end of experiment, placenta was perfused with radioactivity-free buffer for 10 min, excised from the uterine tissue, and dissolved in tissue solubilizer (Solvable; PerkinElmer Life and Analytical Sciences, Boston, MA), and its radioactivity was measured to detect tissue-bound cimetidine.

Two types of perfusion systems were used in this study. For pharmacokinetic analysis of concentration-dependent transplacental passage of cimetidine, both maternal and fetal sides of the placenta were perfused in open-circuit systems, without recirculation of the perfusate. Cimetidine was added to the maternal (in maternal-to-fetal studies) or fetal (in fetal-to-maternal studies) reservoir immediately after successful surgery followed by an approximately 10-min stabilization period before sample collection started (time 0). Fetal effluent was sampled into preweighed vials in 5-min intervals and analyzed for [^{3}H]cimetidine.

To investigate the capability of Bcrp to remove its substrate from fetal circulation, both maternal and fetal sides of the placenta were infused with equal concentrations of cimetidine and after 10-min stabilization period, the fetal perfusate (10 ml) was recirculated for 60 min. Samples (200 μl) were collected every 10 min from the maternal and fetal reservoirs, and [^{3}H]cimetidine concentration was measured. This experimental setup ensures steady cimetidine concentration on the maternal side of the placenta and enables investigations of maternal/fetal concentration ratio; any net transfer of the drug implies transport against a concentration gradient and is evidence for active transport.

Effect of Cimetidine Inflow Concentrations and BCRP Inhibitors on Transplacental Clearance. To investigate the effect of cimetidine concentrations on maternal-to-fetal and fetal-to-maternal clearances, cimetidine and [^{3}H]cimetidine as a tracer were added to the maternal or fetal reservoir, respectively, in one of the following concentrations: 0.005, 0.1, 1, 30, 100, or 1000 μM. The inflowing cimetidine concentration was maintained constant for the duration of the experiment; transplacental clearances of cimetidine were calculated for every concentration from all measured intervals as described below.

To study the effect of BCRP and P-gp inhibitors, 2 μM fumitremorgin, 2 μM GF120918, 10 μM cyclosporine, or 25 μM verapamil was added to the maternal or fetal reservoir in the 10th min of perfusion. Subsequently, transplacental clearance of cimetidine in the period of 0 to 10 min (without inhibitor) was compared with that in 20 to 30 min (with inhibitor), leaving the mid-interval of 10 to 20 min as a stabilization period to achieve a new steady state after addition of inhibitor. This experimental setup allows for direct observations of inhibitor effect in one animal, reducing possible interindividual variability.

Pharmacokinetic Analysis of Efflux Transport Activity in the Placenta. Organ clearance concept was applied to mathematically describe maternal-to-fetal and fetal-to-maternal transport of cimetidine in open-circuit perfusion system. Averaged data from the intervals of 0 to 10 min (control) and 20 to 30 min (inhibitor) of placental perfusions were used for the following calculations. Total maternal-to-fetal transplacental clearance (Cl_{Tmf}) of cimetidine normalized to placenta weight was calculated according to eq. 1.

$$Cl_{Tmf} = \frac{C_{fv} \cdot Q_f}{C_{ma} \cdot w_p} \quad (1)$$

where C_{fv} is the concentration of cimetidine in the umbilical vein effluent, Q_f is the umbilical flow rate, C_{ma} is the concentration of cimetidine in the maternal reservoir, and w_p is the wet weight of the placenta.

In fetal-to-maternal studies, the ability of the placenta to remove cimetidine from the fetal circulation was expressed as extraction ratio (ER) using eq. 2 (Shargel and Yu, 1993):

$$ER = \frac{(C_{fa} - C_{fv})}{C_{fa}} \quad (2)$$

where C_{fa} is the concentration of cimetidine in the fetal reservoir entering the perfused placenta via the umbilical artery. Total fetal-to-maternal clearance normalized to placenta weight (Cl_{Tfm}) was calculated according to eq. 3:

$$Cl_{Tfm} = \frac{ER \cdot Q_f}{w_p} \quad (3)$$

To distinguish between passive and active components of the transplacental movement, the following concept was applied (Fig. 1). Assuming the total transplacental passage of cimetidine being a function of passive diffusion and efflux activity of BCRP, the Cl_{Tmf} and Cl_{Tfm} are described by eqs. 4 and 5, respectively:

$$Cl_{Tmf} = Cl_{pd} - Cl_{efflux} \quad (4)$$

$$Cl_{Tfm} = Cl_{pd} + Cl_{efflux} \quad (5)$$

where Cl_{pd} is clearance of passive diffusion, and Cl_{efflux} expresses the efflux activity of the transporter. Because Cl_{efflux} is a capacity-limited (nonlinear) process, it can be expressed in terms of Michaelis-Menten kinetics:

$$Cl_{efflux} = \frac{V_{max}}{K_m + C_{ma(fa)}} \quad (6)$$

where V_{max} is the maximal velocity of the transport, K_m is the concentration at which half the maximal velocity is reached, and $C_{ma(fa)}$ is substrate concentration in maternal (C_{ma}) or fetal (C_{fa}) circulation.

In maternal-to-fetal studies, adding Cl_{efflux} into eq. 4 yields the following equation, which was used to fit clearance versus inflow concentration data:

$$Cl_{Tmf} = Cl_{pd} - \frac{V_{max}}{K_m + C_{ma}} \quad (7)$$

By analogy, when the effect of fetal inflow concentrations on fetal-to-maternal clearance was investigated, data were fitted by the following equation:

$$Cl_{Tfm} = Cl_{pd} + \frac{V_{max}}{K_m + C_{fa}} \quad (8)$$

Radioactivity remaining in the placental tissue after perfusion was less than 0.4 ± 0.06% of the infused dose in both maternal-to-fetal and fetal-to-maternal studies, regardless of total cimetidine concentration. Therefore, it was ignored in pharmacokinetic modeling. Data were fitted using reciprocal weighting and the numerical module of SAAM II (SAAM Institute, Seattle, WA).

Statistical Analysis. For each group of placental perfusion experiments, the number of animals was $n \geq 4$. Cellular uptake studies are based on $n = 4$. One-way ANOVA followed by Bonferroni's test or Student's t test were used where appropriate to assess statistical significance. Differences of $p < 0.05$ were considered statistically significant.

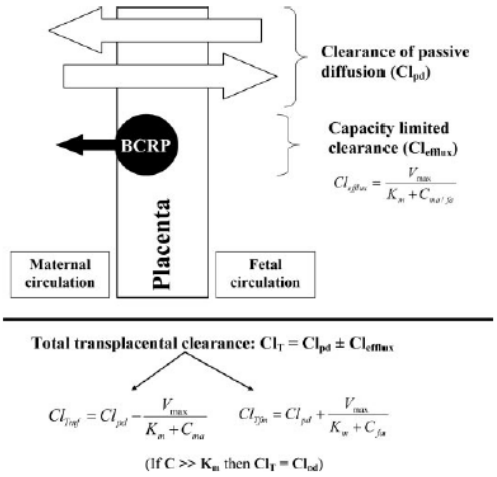

Fig. 1. Schematic depiction of pharmacokinetic analysis applied in this study to evaluate efflux transporter activity in the placenta. This model assumes two processes involved in the transplacental passage: 1) passive diffusion governed by Fick's law (depending mainly on drug's physical-chemical properties, concentration gradient, protein binding, and membrane area and thickness), here described as Cl_{pd} and 2) saturable efflux process governed by the rules of Michaelis-Menten nonlinear kinetics, here described as Cl_{efflux}. Depending on the direction of substrate movement, these two events add up (in fetal-to-maternal direction) or subtract (in maternal-to-fetal direction) to obtain the value of Cl_T. If substrate concentration largely exceeds the Michaelis-Menten constant ($C \gg K_m$), then total placental clearance equals to clearance of passive diffusion. Cl_{Tmf} and Cl_{Tfm} are described under *Pharmacokinetic Analysis of Efflux Transport Activity in the Placenta*.

Results

Expression of Bcrp and P-gp in Rat Placenta and HRP-1 Cells. RT-PCR and Western blotting were used to investigate the expression of Bcrp and P-gp in the rat placenta and HRP-1 cell line and compared with that in kidney as a positive control (Tanaka et al., 2005). The bands corresponding to 329, 331, and 188 bp for *mdr1a*, *mdr1b*, and *Bcrp*, respectively, were visualized under the ultraviolet light (Fig. 2A). Expression of *Bcrp* was detected in rat kidney, placenta, and HRP-1 cell line samples. In contrast, expression of both *mdr1a* and *mdr1b* (coding for P-gp) was detected only in rat kidney and placenta but not in HRP-1 cell line. Likewise, application of polyclonal anti-Abcg2 antibody M-70 revealed significant levels of Bcrp in the rat placenta, kidneys, and HRP-1 cells. Using C219 monoclonal antibody, we confirmed the expression of P-gp in the rat placenta and kidneys only, whereas no signal for P-gp was detected in HRP-1 cell lysate (Fig. 2B).

Immunohistochemical Localization of Bcrp in the Rat Term Placenta. Localization of Bcrp expression in the rat term placenta was investigated by immunohistochemistry at the light microscopy level. Four placentas (gestation day 21) were used for the experiments. The rat placenta is composed of two morphologically different zones, the junctional zone (maternal blood spaces separated by trophoblastic trabeculae that do not contain fetal blood vessels) and the labyrinth zone (maternal blood separated from fetal blood vessels by trophoblast cells). Bcrp was detected in the inner

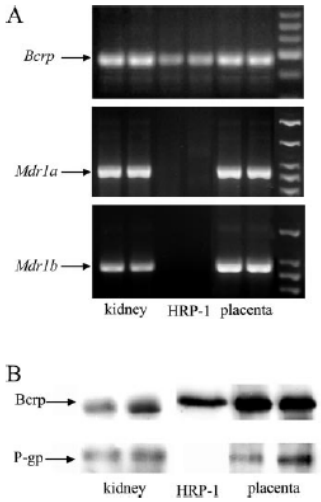

Fig. 2. A, mRNA expression of the rat Bcrp and P-glycoprotein (Mdr1a and Mdr1b) in rat kidney, placenta, and HRP-1 rat placental cell line. B, Western blot immunoanalysis of protein expression of the rat Bcrp and P-gp in the rat placenta and kidney and in the HRP-1 rat trophoblast cell line. Kidneys and placentas were randomly sampled from five female rats and independently processed as described under *Materials and Methods*; two representative samples are shown for each tissue.

layers of the syncytiotrophoblast (layers II and III) of the labyrinth zone only (Fig. 3). No Bcrp staining was visible in either layer I or in the fetal capillaries.

Bcrp Efflux Activity in HRP-1 Cell Line. To investigate Bcrp and/or P-gp Activity in HRP-1 Placental Cells, 500 nM BODIPY FL prazosin as a common substrate of BCRP and P-gp was added to the cells, and the effect of Bcrp and/or P-gp inhibitors was observed. In agreement with gene expression data, only BCRP-specific inhibitor Ko143 (1 μM) and dual BCRP and P-gp inhibitor GF120918 (1 μM) increased BODIPY FL prazosin accumulation by more than 100% ($p < 0.05$). In contrast, P-gp-specific inhibitors PSC833 (1 μM) and verapamil (25 μM) did not affect BODIPY FL prazosin

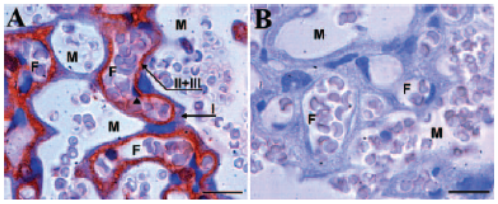

Fig. 3. Immunohistochemical detection of Bcrp in the labyrinth of rat term placenta. Immunohistochemical staining was performed with the monoclonal antibody M-70 (1:10 dilution). A, strong reactivity for Bcrp is visible in the inner layers (II and III) of syncytiotrophoblast. Fetal capillaries or layer I of syncytiotrophoblast do not reveal any positivity. B, for negative control, the slides were treated in the same manner, except nonimmune isotype-matched immunoglobulins were substituted for the primary antibody to Bcrp. F, fetal capillaries; M, maternal blood. Arrowhead shows nucleus of endothelial cell of the fetal capillary, and arrows point to layer I and layers II + III of syncytiotrophoblast (hematoxylin counterstained). Scale bars, 30 μm.

accumulation, suggesting undetectable activity of P-gp in the HRP-1 cell line (Fig. 4).

Consistency of Perfusion Experiments. To determine a steady-state period suitable for inhibitor studies in both maternal-to-fetal and fetal-to-maternal experiments, placenta was first perfused with 0.1 μM cimetidine for 50 min. If no inhibitor was added, we observed steady clearances for the whole period. When a BCRP inhibitor was added to the maternal perfusate in the 10th min of perfusion, it took approximately 5 to 10 min to achieve a new steady state (data not shown). Therefore, to evaluate the effect of inhibitor on cimetidine transplacental passage, samples from the 0- to 10-min interval of perfusion were averaged and compared with those collected in 20- to 30-min interval.

Effect of BCRP and P-gp Inhibitors on Transplacental Passage of Cimetidine. To test interactions of cimetidine with placental Bcrp or P-gp, dual and/or specific inhibitors of these transporters were added to maternal reservoir. Addition of BCRP inhibitors GF120918 (2 μM) or fumitremorgin C (2 μM) caused significant change in transplacental clearance of cimetidine (Fig. 5). Interaction of cimetidine with human P-gp has recently been ruled out using MDR1-transfected Madin-Darby canine kidney II and LLC-PK1 cell lines (Pavek et al., 2005). To exclude any confounding effects of rat P-gp in transplacental passage of cimetidine, P-gp inhibitors cyclosporine (10 μM) or verapamil (25 μM) were tested. Although these compounds increased maternal-to-fetal clearance of rhodamine123, a P-gp substrate, in our previous study (Pavek et al., 2003), they did not interfere with transport of cimetidine. Therefore, transplacental passage of cimetidine does not seem to be affected by P-gp, and cimetidine can be used as a marker compound to functionally analyze efflux activity of Bcrp in the rat placenta. Inhibitory effect observed after addition of GF120918 may be ascribed to Bcrp blockade only, although we cannot exclude possible contribution of other, yet unidentified GF120918-sensitive cimetidine transporter.

Effect of Inflow Cimetidine Concentrations on Transplacental Clearance. Cimetidine was infused to maternal or fetal side of the placenta at one of the following concentrations: 0.005, 0.1, 1, 30, 100, or 1000 μM. In both maternal-to-fetal and fetal-to-maternal transport studies, increase in cimetidine concentration caused significant change in transplacental clearance, confirming nonlinearity of the process and involvement of a capacity-limited mechanism (Figs. 6 and 7). Furthermore, addition of a BCRP inhibitor significantly affected clearances at lower cimetidine concentrations, whereas at concentrations above 30 μM, inhibitor was rather ineffective.

Fitting experimental data with eqs. 7 and 8 provided pharmacokinetic parameters describing passive and Bcrp-mediated transplacental passage of cimetidine (Table 2). It is evident that passive movement across the placenta (described by Cl_{pd}) is independent of direction and of inhibitor used. Conversely, Cl_{efflux} is a concentration- and inhibitor-dependent parameter. At substrate concentrations largely exceeding the Michaelis-Menten constant ($C \gg K_m$), the transporter is saturated, the nonlinear fraction of eqs. 7 and 8 approaches zero, and both equations are reduced to linear processes only [total transplacental clearance (Cl_T) = Cl_{pd}]; under these conditions, transplacental pharmacokinetics is beyond any quantifiable effect of efflux transporter and is

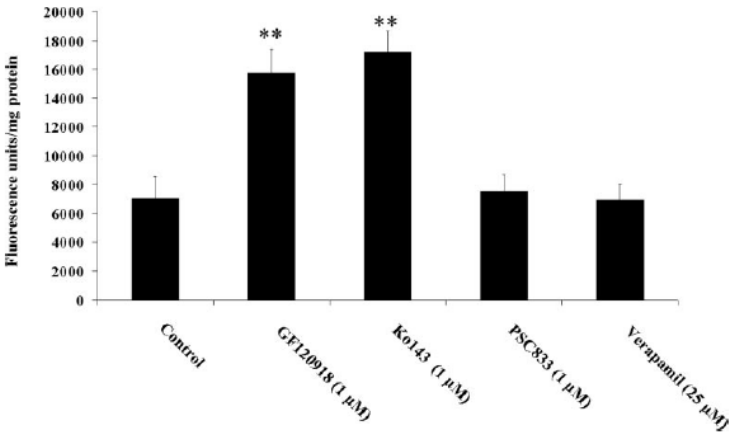

Fig. 4. Uptake of common BCRP and P-gp substrate BODIPY FL prazosin by rat placental HRP-1 cells. BODIPY FL prazosin was added to cells without inhibitor (control) or in the presence of BCRP or P-gp inhibitors. Both GF120918 and Ko143 (BCRP inhibitors) significantly increased accumulation of BODIPY FL prazosin in the cells. P-gp-selective inhibitors PSC 833 and verapamil had no effect on BODIPY FL prazosin accumulation. Data are presented as means ± S.D. of four experiments. One-way ANOVA followed by Bonferroni's test was used; **, $p < 0.05$ compared with control.

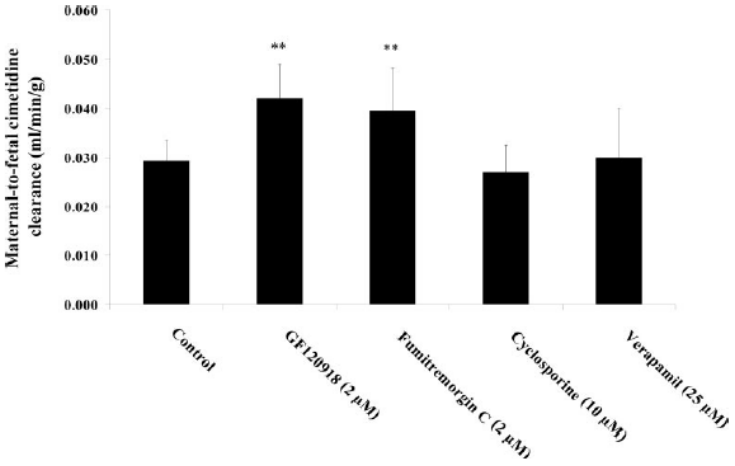

Fig. 5. Effect of Bcrp and P-gp inhibitors on maternal-to-fetal clearance of cimetidine. Cimetidine and [^{3}H]cimetidine tracer were added to the maternal compartment at a concentration of 0.1 μM, and its radioactivity was measured in fetal venous outflow. Total transplacental clearance was calculated by eq. 1 (see *Materials and Methods*). Inhibitor was added to the maternal perfusate in the 10th min. Only BCRP inhibitors GF120918 and fumitremorgin C affected transplacental clearance of cimetidine, whereas P-gp inhibitors cyclosporine and verapamil had no significant effect. Data are presented as means ± S.D. of six experiments. One-way ANOVA followed by Bonferroni's test was used; **, $p < 0.05$ compared with control.

governed exclusively by passive diffusion. This has been experimentally achieved in both maternal-to-fetal and fetal-to-maternal studies when cimetidine concentration was increased to 1000 μM. Furthermore, because addition of inhibitor caused no change in transplacental clearance of 1000 μM cimetidine (Figs. 6 and 7), it is reasonable to assume that at high substrate concentrations, drug-drug interactions will have no effect on penetration of BCRP substrates through placenta.

Comparing maternal-to-fetal and fetal-to-maternal clearances revealed great asymmetry in favor of fetal-to-maternal direction. This was most evident at low cimetidine concentrations (0.005 μM), where fetal-to-maternal clearance was almost 25 times higher ($p < 0.05$) than that in the opposite direction (Fig. 8). At a concentration of 1000 μM, however, both maternal-to-fetal and fetal-to-maternal clearances of cimetidine reached identical values of 0.042 ml/min/g, confirming saturation of Bcrp and limited role of its efflux activity. This experimental value corresponds well with the calculated clearance of passive diffusion (Cl_{pd} ~0.041–0.043 ml/min/g; Table 2).

Bcrp Transports Cimetidine from Fetus to Mother against a Concentration Gradient. To investigate the potential of Bcrp to remove its substrate from fetal circulation, cimetidine was added to both maternal and fetal reservoirs at equal concentrations of 0.005 or 1000 μM and fetal perfusate was recirculated. At a low drug concentration (0.005 μM), cimetidine in the fetal circulation steadily decreased and stabilized after approximately 40 min of perfusion. Fetal-to-maternal concentration ratio reached a value of 0.49 toward the end of the experiment. Decrease in fetal cimetidine was blocked by co-infused BCRP inhibitors (GF120918 or fumitremorgin C; Fig. 9A). At a high cimetidine concentration (1000 μM), maternal and fetal concentrations remained unchanged throughout the perfusion period with fetal/maternal concentration ratio staying close to 1

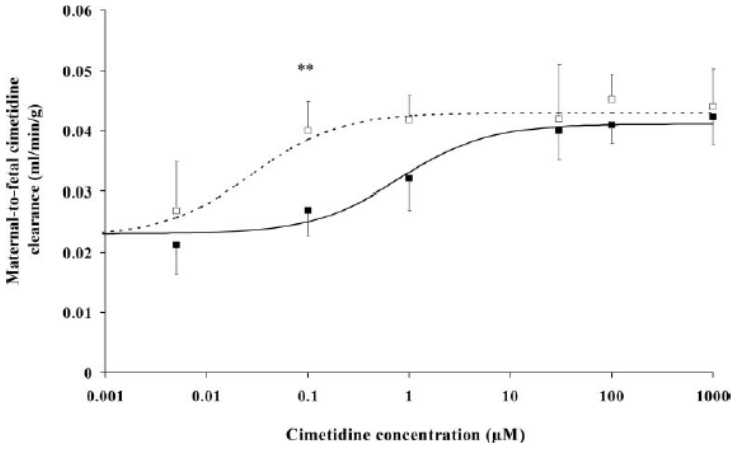

Fig. 6. Transport of cimetidine across the dually perfused rat placenta in maternal-to-fetal direction. Cimetidine with [^{3}H]cimetidine tracer was added to the maternal reservoir, and its radioactivity was measured in fetal venous outflow. Total transplacental clearance was calculated by eq. 1 (see *Materials and Methods*). Changes of clearance with increasing cimetidine concentration confirm nonlinearity of the event and involvement of a saturable mechanism. Inhibitor (2 μM GF120918) was added to block Bcrp activity. At the highest cimetidine concentration tested (1000 μM), clearance reached the value of 0.042 ml/min/g, and inhibitor activity was negligible. Experimental values are presented as means ± S.D. of at least four experiments; the lines represent the best fit of these data to eq. 7. Note the sigmoid shape of the lines with the lower plateau delineating combined effect of passive clearance and efflux activity of Bcrp and the upper plateau representing clearance of passive diffusion alone. Asterisks indicate significant ($p < 0.05$) effect of inhibitor. ■, cimetidine without inhibitor; □, cimetidine with GF120918.

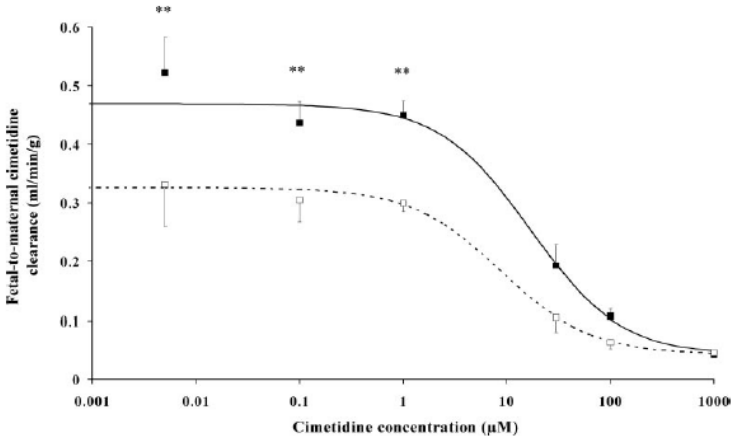

Fig. 7. Transport of cimetidine across the dually perfused rat placenta in fetal-to-maternal direction. Cimetidine with [^{3}H]cimetidine tracer was added to the fetal reservoir, and its radioactivity was measured in fetal venous outflow. Total transplacental clearance was calculated by eq. 3 (see *Materials and Methods*). Changes of clearance with increasing cimetidine concentration confirm nonlinearity of the event and involvement of a saturable mechanism. Inhibitor (2 μM GF120918) was added to block Bcrp activity. As in maternal-to-fetal transport, at the highest cimetidine concentration tested (1000 μM), clearances reached the value of 0.042 ml/min/g, and inhibitor activity was negligible. Experimental values are presented as means ± S.D. of at least four experiments; the lines represent the best fit of these data to eq. 8. Note the sigmoid shape of the lines with the top plateau delineating combined effect of passive clearance and efflux activity of Bcrp and the bottom plateau representing clearance of passive diffusion alone. Asterisks indicate significant ($p < 0.05$) effect of inhibitor. ■, cimetidine without inhibitor; □, cimetidine with GF120918.

(Fig. 9B). These findings demonstrate the potency of placental Bcrp to remove, in a capacity-limited manner, its substrate from fetal compartment and to establish a concentration gradient between maternal and fetal circulations.

Discussion

Detailed knowledge of transplacental kinetics of drugs is essential with respect to fetal safety, fetal medication, and drug-drug interactions during pregnancy. Apart from physical-chemical properties, placental passage of many drugs is controlled by interactions with biotransformation enzymes and/or efflux transporters. It is widely thought that enzymes and drug efflux transporters form an active component of the placental barrier that helps protect fetus against maternal toxins (Marin et al., 2004). In addition, it seems plausible, that these proteins may, to at least some extent, actively metabolize/transport compounds already present in the fetal circulation. Using rat placental perfusion, we have previously demonstrated that 11β-hydroxysteroid dehydrogenase type 2 metabolizes both maternal and fetal corticosterone with a comparable potency (Staud et al., 2006). Likewise, P-gp has been confirmed to favor fetal-to-maternal transport

of its substrates in perfused rat (Pavek et al., 2003) or human placenta (Molsa et al., 2005; Sudhakaran et al., 2005). In the present study, we focused on functional analysis of the latest of ABC drug efflux transporters, Bcrp, using dually perfused rat term placenta and rat placenta-derived cell line HRP-1.

To date, only a few studies have reported on BCRP activity in placenta, and these are mainly based on in vitro models. Very recently, Kolwankar et al. (2005) used placental microvillous membrane vesicles to confirm BCRP function in the human placenta. Subsequently, we have described BCRP expression, localization, and function in an in vitro placental model, the BeWo cell line (Ceckova et al., 2006). In the present study, we used rat placental HRP-1 cell line derived from placental labyrinth region at mid-gestation (Soares et al., 1987). This cell line has previously been used to study several aspects of placental physiology (Soares et al., 1989; Shi et al., 1997; Morris Buus and Boockfor, 2004), metabolism (Xu et al., 2005), or nutrient transport (Novak et al., 2001; Zhou et al., 2003). To our knowledge, however, no studies have been performed so far to investigate expression and/or activity of ABC drug efflux transporters in this in vitro placental model. Bcrp expression was revealed at both mRNA and protein levels. Surprisingly, we did not detect any expression of *mdr1a* or *mdr1b* genes coding for rat P-gp, neither did we find any signal by Western blotting. Consistent with gene and protein expression results, uptake studies revealed only Bcrp activity, whereas P-gp did not affect cell accumulation of BODIPY FL prazosin. These data are similar to what has previously been observed in human choriocarcinoma cell line BeWo (Atkinson et al., 2003; Ceckova et al., 2006; Evseenko et al., 2006) where only BCRP was found to be functionally expressed, whereas P-gp activity was negligible. Lack of expression and function of P-gp in the HRP-1 cell line makes this model inappropriate to investigate the transplacental transport of P-gp substrates. In contrast, it may well serve as a tool to study Bcrp role in transplacental pharmacokinetics, because its efflux activity will not interfere with that of P-gp.

The only functional in vivo studies on Bcrp activity in the placenta so far have been performed in transgenic mice (Jonker et al., 2000, 2002); however, detailed evaluation of BCRP role in transplacental pharmacokinetics is still lacking. In the present study, cimetidine was used to comprehensively describe the role of Bcrp in maternal-to-fetal and fetal-to-maternal transport. Cimetidine was chosen as a model substrate for its convenient properties; it is a BCRP substrate that is not recognized by human P-gp (Pavek et al., 2005), it weakly binds to plasma proteins, and its biotransformation by placental enzymes is negligible (Schenker et al., 1987). In addition, cimetidine passive diffusion through biological membranes is delayed by its physical-chemical properties as shown in transepithelial passage (Pavek et al., 2005) or placental transport (Ching et al., 1987; Schenker et al., 1987); this seems to be an important feature to study substrate interactions with an efflux transporter (Eytan et al., 1996; Lentz et al., 2000).

The localization of Bcrp on the apical, maternal-facing membrane of the rat placenta closely resembles that of P-gp described in our previous studies (Pavek et al., 2003; Novotna et al., 2004). This finding suggests that Bcrp is, like P-gp, important in preventing entry of potential toxins into the fetal compartment. This assumption has been functionally validated in the present study by means of rat perfused placenta: at low cimetidine concentrations (0.005 μM), maternal-to-fetal clearance was 25-fold lower than clearance in the opposite direction. These data confirm that Bcrp causes asymmetry in transplacental clearances in rats by returning substrates coming from maternal side and facilitating transport of drugs from fetus to mother. Interestingly, pharmacokinetic modeling revealed Michaelis-Menten constant for fetal-to-maternal direction to be 20 times higher than that for

TABLE 2

Pharmacokinetic parameters of transplacental passage of cimetidine

Pharmacokinetic parameters were obtained by fitting experimental data with eqs. 7 and 8. GF120918 (2 μM) added to the maternal compartment was used as an inhibitor.

	Maternal-to-Fetal Transport		Fetal-to-Maternal Transport	
	Control	Inhibitor	Control	Inhibitor
Cl_{pd} (ml/min/g)	0.041	0.043	0.042	0.042
V_{max} (nmol/min/g)	0.014	0.00057	7.14	2.47
K_m (μM)	0.80	0.028	16.7	8.71

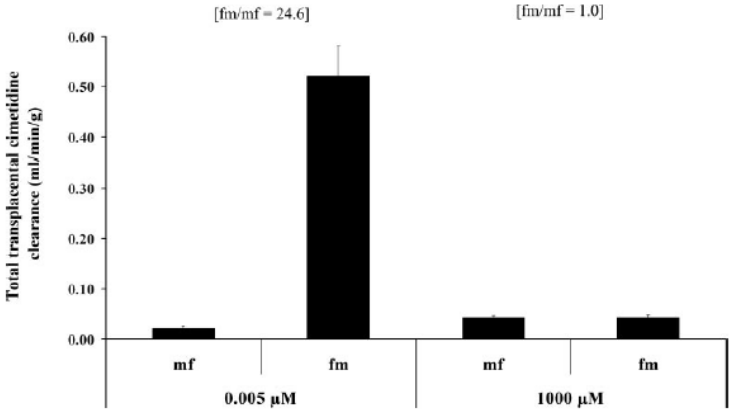

Fig. 8. Ratio of clearances between fetal-to-maternal (fm) and maternal-to-fetal (mf) directions. Cimetidine with [^{3}H]cimetidine tracer was added to the maternal or fetal compartment, and its radioactivity was measured in fetal venous outflow. Total transplacental clearances were calculated by eq. 1 or 3, respectively (see *Materials and Methods*). At low substrate concentrations (0.005 μM), Bcrp efflux activity caused almost 25 times higher clearance in fetal-to-maternal direction. At high substrate concentrations (1000 μM), however, this ratio equalized. Numbers in parentheses show ratio of fm-to-mf clearance.

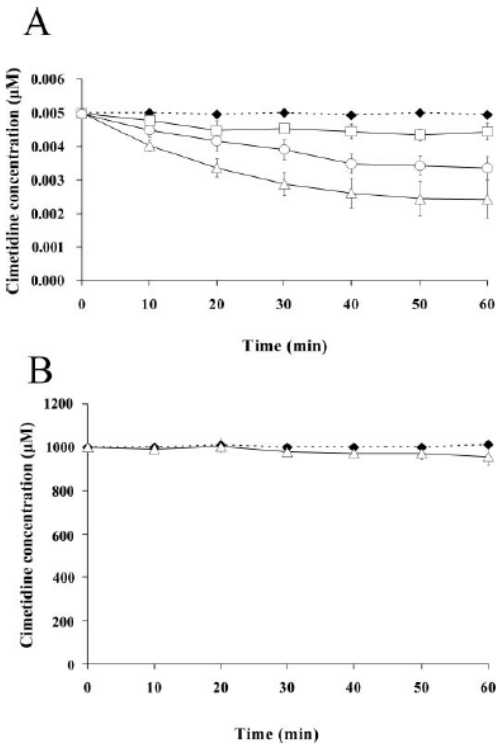

Fig. 9. Elimination of cimetidine from the fetal circulation by placental Bcrp. Cimetidine and [^{3}H]cimetidine tracer were added to both maternal (closed symbol) and fetal (open symbols) circulations at equal concentrations. Fetal perfusate (10 ml) was recirculated and sampled for 60 min. At low cimetidine concentrations of 0.005 μM (A), fetal cimetidine decreased from 0.005 μM down to 0.0024 μM and stabilized after 40 min of perfusion. This decrease was inhibited by both BCRP inhibitors GF120918 (2 μM) and fumitremorgin C (2 μM). At high cimetidine concentrations of 1000 μM (B), no decrease in fetal compartment was observed, suggesting saturation of the transporter. Data are presented as means ± S.D. of three experiments. ♦, maternal cimetidine concentration; □, fetal cimetidine concentration with GF120918; ○, fetal cimetidine concentration with fumitremorgin C; △, fetal cimetidine concentration without inhibitor.

maternal-to-fetal direction. We assume this difference is caused by polarized localization of Bcrp on the maternal side of the placenta; as a result, a compound administered to the fetal circulation needs to pass through fetal tissues to reach the transporter. This suggests that much higher cimetidine concentrations are needed to saturate Bcrp transporter during fetal-to-maternal passage than in the opposite direction.

To investigate the potential of Bcrp to remove drugs already present in the fetal compartment, both maternal and fetal sides of the placenta were perfused with equal concentrations of cimetidine and fetal perfusate was recirculated. After short equilibration period, we observed significant decrease in fetal cimetidine concentrations, confirming that Bcrp can actively remove its substrate from the fetal compartment. Because decrease in cimetidine concentration continued even at later intervals (dropping by more than 50% within 60 min of perfusion), it is evident that Bcrp in rats can pump this compound from fetus to mother even against a concentration gradient.

Interestingly, several studies on cimetidine placental transfer were published two decades ago with intriguing results. When studied in sheep, large cimetidine gradient between mother and fetus was observed (Mihaly et al., 1983). In a follow-up study, the authors suggested that an active transporter from the fetal to the maternal circulation might be responsible for this discrepancy (Ching et al., 1985). In contrast, when investigated in the dually perfused human placenta, two papers concluded that transport of cimetidine was very slow and occurred by passive diffusion with lack of saturation kinetics (Ching et al., 1987; Schenker et al., 1987). These contrasting findings might be explained by interspecies differences; however, one has to realize that these studies were performed before efflux transporters were discovered and described, with limited range of cimetidine concentrations, and without the option to use appropriate inhibitors. Therefore, possible role of a drug efflux transporter in the transplacental pharmacokinetics of cimetidine could not have been taken in account. Our present findings suggest that Bcrp is the transporter responsible for limited maternal-to-fetal passage and large maternal/fetal concentration ratio of cimetidine in rats. However, BCRP activity in perfused placentas of other species must be elucidated before a final conclusion is drawn.

Regarding BCRP expression in human tissues, relatively high mRNA levels were observed in placenta, liver, and small intestine with lower expression in the kidney, heart, and brain (Doyle et al., 1998). In rodents, however, a different mRNA distribution pattern was indicated by Tanaka et al. (2005). They found high expression levels in kidney and small and large intestine, and lower levels were found in other tissues, including brain and placenta. Based on these observations, the authors suggested limited importance of placental Bcrp in rodents (Tanaka et al., 2005). In contrast, functional role of placental Bcrp has been proposed in mice by Jonker et al. (2000, 2002) and thoroughly assessed in the rat placenta in our study. Therefore, mRNA expression levels do not have to necessarily correlate with transport potency of the protein, because there are a number of other factors that determine its functional activity, such as post-transcriptional/post-translational modifications in protein expression as well as strategic localization of BCRP along the maternal interface.

In conclusion, functional expression of Bcrp in the rat placenta and rat placental HRP-1 cell line was confirmed in this study. A pharmacokinetic model was applied to distinguish between passive and Bcrp-mediated placental transport of cimetidine as a model substrate. We provide evidence for striking asymmetry between maternal-to-fetal and fetal-to-maternal transport of cimetidine; this difference is partly lowered by addition of BCRP inhibitors and abolished at high substrate concentrations. In addition, using closed perfusion system on the fetal side of the placenta, we are the first to demonstrate that Bcrp, despite being localized on the maternal-facing side, actively removes cimetidine from the fetal circulation against concentration gradient. Based on our findings, we propose a two-level defensive role of placental BCRP in which the transporter 1) reduces passage of its

III.

RECIPROCAL CHANGES IN MATERNAL AND FETAL METABOLISM OF CORTICOSTERONE IN RAT DURING GESTATION

Reciprocal Changes in Maternal and Fetal Metabolism of Corticosterone in Rat During Gestation

K. Vagnerová, PhD, Z. Vacková, MSc, P. Klusoňová, MSc, F. Štaud, PhD, M. Kopecký, PhD, P. Ergang, MSc, I. Mikšík, PhD, and J. Pácha, PhD

Objective. The objective of this study was to investigate the role of 11β-hydroxysteroid dehydrogenases (11HSD1 and 11HSD2) in determining the fetal concentration of glucocorticoids. Methods. The expression patterns for mRNA abundance, protein level, and enzyme activities of placental and fetal 11HSD1 and 11HSD2 were assessed from embryonic day 13 (E13) to day 21 (E21; term E22). The transplacental passage of maternal corticosterone and its contribution to fetal glucocorticoids was also studied. Results. Placental 11HSD1 mRNA decreased between days E13 and E14 and then remained at much lower values up to E21. Similarly, NADP+-dependent 11β-oxidation and 11-reduction were lower in late gestation. In contrast, placental 11HSD2 mRNA and protein decreased between E13 and E21. Dithiothreitol increased the activity of 11HSD2 and the output of 11-dehydrocorticosterone into fetal circulation. The fetal activity of 11HSD1 increased and 11HSD2 decreased between E16 and E21. Conclusions. The final third of gestation is accompanied by reciprocal changes in placental and fetal metabolism of corticosterone due to changes in 11HSD1 and 11HSD2 not only at the level of transcription but also at a posttranslational level.

KEY WORDS: Placenta, fetal development, metabolism of glucocorticoids, corticosterone.

Glucocorticoids are hormones that play an important regulatory role during the maturation of fetal organs and the timing of parturition.[1,2] Fetal glucocorticoids can come from several sources, in particular through glucocorticoid secretion by the fetal adrenal glands after the activation of the fetal hypothalamic–pituitary–adrenal axis,[2] by the local production of glucocorticoids in the fetal membranes[3] and by the transplacental transfer of maternal glucocorticoids to the fetus.[4-6] Although steroids can be considered to cross the placenta easily, the levels of glucocorticoid hormones are much lower in fetuses than in maternal blood for much of gestation.[7-9] The difference between the maternal and fetal plasma concentration of glucocorticoids is ascribed to the placenta. It protects the fetuses against maternal cortisol or corticosterone by oxidation of these hormones to their biologically inactive 11-oxo derivatives cortisone and 11-dehydrocorticosterone via the activity of the enzyme 11β-hydroxysteroid dehydrogenase (11HSD).[10] Importantly, mutations in the gene of this enzyme in humans are associated with low birth weight and exposure of pregnant rats to blockers that inhibit the activity of this enzyme reduces offspring birth weight and produces long-term metabolic and neuroendocrinic changes that lead to increases in adult blood pressure, glucose levels, and hypothalamic–pituitary–adrenal axis activity, that is, prenatal exposure to excess glucocorticoids might link fetal growth with adult pathophysiology.[11]

Two functionally distinct types of 11HSD exist—the NAD^+-dependent enzyme (11HSD2), which operates only as an oxidase and catalyzes the conversion of cortisol and corticosterone to cortisone, and 11-dehydrocorticosterone and the $NADP^+$/NADPH-dependent enzyme (11HSD1), which has both oxidase and reductase activities

From the Institute of Physiology, Czech Academy of Sciences, Prague (KV, PK, MK, PE, IM, JP) and Faculty of Pharmacy, Charles University, Hradec Králové (ZV, FS, MK), Czech Republic.

This study was supported by grants KJB5011402 and AV0Z50110509 from Academy of Sciences and by grant no. 102/2006/C/FaF from the Grant Agency of Charles University.

Address correspondence to: J. Pácha, Institute of Physiology, Czech Academy of Sciences, Vídeňská 1083, 142 20 Prague 4–Kr?, Czech Republic. E-mail: pacha@biomed.cas.cz.

Reproductive Sciences Vol. 15 No. 9 November 2008 921-931
DOI. 10.1177/1933719108319161

and catalyzes the bidirectional interconversion between cortisol and cortisone or corticosterone and 11-dehydrocorticosterone. The expression of both isoforms has been demonstrated and characterized to varying degrees in human,[4,12,13] baboon,[14,15] rat,[16-18] mouse,[19,20] and porcine placenta.[21]

In rat placenta, both 11HSD1 and 11HSD2 are expressed in a developmentally programmed manner in 2 functionally distinct placental zones—the labyrinth zone in which both maternal and fetal vessels are found and in the basal zone, located deeper than the labyrinth.[17,18] The coexpression of both types of 11HSDs raise the possibility that the placenta is able to change its placental metabolic capacity not only to exclude maternal glucocorticoids from the fetus but also to modulate the access of maternal glucocorticoids to the fetal compartment. In addition, our recent data indicate that rat placenta forms a potent but incomplete barrier to maternal corticosterone.[6] However, the definitive role of the placenta in the translocation of maternal corticosterone throughout gestation remains unknown, as does the relationship between placental and fetal 11HSD metabolism in determination the ratio of active and biologically inert glucocorticoids in fetal circulation. Therefore, this study was undertaken to explore the effect of placental and fetal glucocorticoid metabolism on fetal plasma glucocorticoids. For this purpose, we assessed the developmental patterns of 11HSD1 and 11HSD2 together with the transplacental transfer of maternal corticosterone and its contribution to fetal glucocorticoids.

MATERIALS AND METHODS

Animals

Wistar rats (Institute of Physiology, Czech Academy of. Sciences, Prague) were housed in a room with a controlled light cycle (12-hour light/12-hour dark) and temperature (22°C) with free access to standard laboratory chow and tap water. The females were mated with a male for 1 night and the next day was taken as day 0 of pregnancy if spermatozoa were found in the vaginal smears. Placentae were used from rats on days E13, E14, E16, E19, and E21 of gestation. The rats used for infusion experiments in vivo and for dually perfused placenta in vitro were fasted before the experiments and anesthetized with pentobarbital (Nembutal; Abbott Laboratories, North Chicago, IL) in a 40 mg/kg dose administered into the tail vein. All other rats were killed by cervical dislocation and the placenta removed, free of fetal membrane. The procedures were approved by the Czech Academy of Sciences Animal Care and Use Committee and the Ethical Committee of the Faculty of Pharmacy, Charles University, and were carried out in accordance with the Guide for the Care and Use of Laboratory Animals, and the European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes.

Transplacental Passage of [^{3}H]Corticosterone in Rats In Vivo

To evaluate the transplacental passage of corticosterone and the role of 11HSD in this process, infusion studies using [^{3}H]corticosterone were carried out on days E16 and E21 of gestation. The carotid artery and jugular vein were cannulated for blood sampling and isotope infusion, respectively. Rats received a priming dose of [^{3}H]corticosterone (5 µCi/0.5 mL 0.9% NaCl) and were then infused with [^{3}H]corticosterone (0.5 µCi/min) for 60 minutes. Preliminary experiments indicated the radioactivity in blood plasma of the jugular vein remained constant after 30 minutes and this time was defined as the sample time when steady-state concentration had been attained. Hence, blood and fetus samples were taken for further analysis 60 minutes after the onset of the isotope infusion and 30 minutes after steady-state concentration was reached. The blood was collected into heparinized tubes, centrifuged at 3000 × *g* for 10 minutes and the plasma stored at −70°C until analyzed. Immediately after blood sampling the fetuses were dissected, frozen in liquid nitrogen and stored at −70°C until analysis. Estimation of the total radioactivity was done in maternal blood samples and in homogenates of fetuses treated with Solvable tissue solubilizer (Perkin Elmer, Welleslay, MA).

To determine the corticosterone/11-dehydrocorticosterone ratio, the samples were extracted with ethyl acetate. Briefly, internal standard [^{3}H]progesterone and radioinert corticosterone, 11-dehydrocorticosterone, and progesterone were added to the samples of blood plasma and tissue homogenate (in 0.1 mol/L phosphate-buffered saline), mixed, and extracted with ethyl acetate (1 vol sample/5 vol solvent). The mixture was centrifuged at 3000 × *g*, the organic layer removed, washed with 0.1 mol/L NaOH, then with water, centrifuged again and the supernatant transferred to a glass vial. The extraction of the sample was repeated 3 times, the organic layers were combined, dried under nitrogen at 37°C and stored at −20°C until high-performance liquid chromatography (HPLC) analysis.

Dual Perfusion of Rat Placenta

To investigate the effect of dithiotreitol (DTT) on the conversion capacity of 11HSD2, the method of dually perfused rat placenta from day 21 of gestation was used as described previously.[6] Briefly, the placenta was excised and submerged in heated Ringer saline. A catheter was inserted into the uterine artery proximal to the blood vessel supplying the selected placenta and connected with a peristaltic pump. Krebs' perfusion liquid containing 1% dextran was brought from the maternal reservoir at a rate of 1 mL/min. The uterine vein, including the anastomoses to other fetuses, was ligated behind the perfused placenta and cut so that maternal solution could leave the perfused placenta. The selected fetus was separated from the neighboring ones by ligatures. The umbilical artery was catheterized using a 24-gauge catheter and connected with the tubing through which the fetal perfusion liquid from the fetal reservoir was supplied at a rate of 0.5 mL/min. The umbilical vein was catheterized in a similar manner and the selected fetus was removed. Before the start of each experiment, the fetal vein effluent was collected into preweighed glass vials to check for possible leakage of perfusion solutions from the placenta. Where leakage was found the experiment was terminated. Maternal and fetal perfusion pressures were monitored continuously throughout the perfusion experiments.

Corticosterone and [^{3}H]corticosterone as a tracer were brought to the perfused placenta via the uterine artery immediately after catheterization. The final concentration of corticosterone in the maternal reservoir was 50 nmol/L, which corresponds to the physiological values of unbound corticosterone in rat during the last week of gestation.[22] After a 10-minute delay to achieve steady-state conditions, samples were collected at 10-minute intervals from the fetal umbilical vein. After the first sample was collected, DTT was added to the maternal reservoir to a concentration of 0.5 or 5 mmol/L. After a 10-minute delay to reestablish steady state, collection of the second 10-minute interval sample was initiated. Samples were analyzed for [^{3}H]corticosterone and [^{3}H]11-dehydrocorticosterone by HPLC. The conversion capacity of placental 11β-oxidation was expressed as the ratio of 11-dehydrocorticosterone (metabolite) concentration to the concentration of total corticosteroids (corticosterone and 11-dehydrocorticosterone) in the fetal effluent.

Measurement of 11HSD Activity in Tissue Homogenates

Whole placentae or the whole fetuses of different days of gestation were homogenized in ice-cold buffer containing 200 mmol/L sucrose and 10 mmol/L Tris/HCl at pH 8.5 (1:9 weight/vol) using a Polytron homogenizer (Kinematica AG, Littau, Switzerland). The homogenates were centrifuged at 1000 × *g* for 10 minutes at 4°C, the protein concentration determined by the Coomassie blue method, and the homogenates used immediately for the measurement of 11HSD activity.

11HSD2 activity was measured as the NAD^+-dependent oxidation of corticosterone in a radiometric conversion assay as reported previously.[23] Briefly, the conversion of [^{3}H]corticosterone (final concentration 14.5 nmol/L) to [^{3}H]11-dehydrocorticosterone was measured in an incubation buffer (100 mmol/L KCl, 50 mmol/L Tris/HCl; pH 8.5) containing 400 µmol/L NAD^+. In some experiments the incubation buffer also contained a reducing agent, DTT at concentrations of 0.5 to 10.0 mmol/L. 11HSD1 was measured as the conversion of [^{3}H]11-dehydrocorticosterone to [^{3}H]corticosterone in the presence of 400 µmol/L NADPH and a NADPH-regenerating system (1.3 mmol/L glucose-6-phosphate, 2 U glucose-6-phosphate dehydrogenase) or as the oxidation of [^{3}H]corticosterone to [^{3}H]11-dehydrocorticosterone in the presence of 400 µmol/L $NADP^+$. In both cases, the incubation buffer contained 100 mmol/L KCl, 50 mmol/L Tris/HCl and was pH 7.5. The velocities of the reaction were linear within the periods of incubation used. The reactions were stopped by cooling and the samples were extracted with a Sep-Pak C_{18} cartridge and stored. The steroids were quantified by HPLC.

Analysis of Steroids

The separation and quantification of unlabelled and [^{3}H]-labeled steroids were conducted by HPLC as described previously.[6,24] Briefly, the steroids were analyzed by an Agilent HPLC 1100 system (Agilent, Palo Alto, CA) with a Lichrospher 100 RP-18 column (125 × 4 mm; Merck, Darmstadt, Germany) and linear methanol–water gradient from 42:58 (vol/vol) to 62:38 (vol/vol) at 15 minutes followed by isocratic washing with 100% methanol for 10 minutes. The flow rate was 1.0 mL/min and the column temperature was held at 45°C. An Agilent 1100 ultraviolet absorbance detector

Table 1. Primers Used for Reverse Transcriptase-Polymerase Chain Reaction

	Forward	Reverse
11HSD1	GAGTTCAGACCAGAAATGCTCC	TGTGTGATGTGATTGAGAATGAGC
11HSD2	CCGGTTGTGACACTGGTTTTG	GGGGTATGGCATGTCTCCTG
β-Actin	CCGTAAAGACCTCTATGCCA	AAGAAAGGGTGTAAAACGCA

(at 254 nm) and Radiomatic 150 TR radioactivity detector (Packard, Downers Grove, IL) with a flow cell and Ultima-Flo M scintillation cocktail (Perkin Elmer, Boston, MA; flow rate 3 mL/min) were used for the detection and quantification of non-radioactive and radioactive steroids, respectively.

Reverse Transcription and Real-Time RT-PCR

Total RNA was extracted from frozen placenta samples by the guanidine thiocyanate method. To remove contamination by genomic DNA, the samples of isolated RNA were treated with DNase (Promega, Madison, WI) following the standard protocol. First strand cDNA was synthesized using 5 µg of RNA and M-MLV (Invitrogen, Lofer, Austria) as described earlier.[6] Quantitative reverse transcriptase polymerase chain reaction (RT-PCR) was used to determine the mRNA abundances of 11HSD1, 11HSD2, and β-actin using a Fast Start DNA Master Sybr Green I kit (Roche, Mannheim, Germany), LightCycler instrument (Roche) and the primers listen in Table 1. The PCR was performed in duplicates in a total volume of 10 µL containing 1 µL of 10-fold diluted cDNA; 3 mmol/L (11HSD1), 4 mmol/L (11HSD2), or 5 mmol/L (β-actin) $MgCl_2$; 0.5 µmol/L of each primer and PCR reaction mix (1×). The LightCycler was programmed as follows: preincubation and denaturation of the template cDNA for 10 minutes at 95°C followed by 45 cycles of amplification at 95°C for 15 seconds, 55°C for 10 seconds (11HSD1, β-actin), or 60°C for 5 seconds (11HSD2), 72°C for 15 seconds (11HSD1, β-actin) or 20 seconds (11HSD2). A melting analysis was performed after each elongation. Quantification was performed using standard curves for each pair of primers from serial dilutions of kidney cDNA containing all 3 transcripts. The results were calculated as the relative expression of 11HSD1 mRNA or 11HSD2 mRNA to β-actin mRNA.

Western Blotting Analysis

Western blots were performed as described previously.[6] Briefly, the placenta and kidney (which acted as a positive control) were homogenized, centrifuged at 400 × *g* for 10 minutes and the supernatant centrifuged again at 100 000 × *g* for 60 minutes. The pellets were resuspended and sonicated in Laemmli buffer containing 2% β-mercaptoethanol, boiled for 3 minutes and then stored at −20°C until used for electrophoretic analysis.

The proteins were separated by 10% SDS-polyacrylamide gel electrophoresis and electroblotted in a semidry blotting system. The blots were incubated with sheep anti-rat 11HSD2 polyclonal antibody (Chemicon International, Inc, Temecula, CA) and subsequently incubated with peroxidase-conjugated rabbit anti-sheep immunoglobulin G (Pierce Biotechnology, Inc, Rockford, IL). The protein bands were visualized using Super Signal West Femto substrate (Pierce) and detected with a LAS-1000 luminiscence analysing system (Fuji, Tokyo, Japan). The band of approximately 40 kDa, corresponding to the known molecular mass of rat 11HSD2 was clearly visible in all specimens tested.

Despite the clear expression of 11HSD1 mRNA and 11-reductase activity in placental tissue, no specific signal for the 11HSD1 protein was measurable by immunoblotting at any stage of gestation examined, although several commercially accessible anti-rat 11HSD1 antibodies were tested (Alpha Diagnostics, Cayman Chemicals, Santa Cruz Biotechnology, US Biological) and some of them gave a positive signal with rat liver.

Immunocytochemistry of Placental 11HSD2

Sheep anti-rat polyclonal antibody raised against 11HSD2 was purchased from Chemicon Int. (Temecula, CA). Rat placentae were fixed in 4% paraformaldehyde (4°C) at pH 7.4 (Sigma-Aldrich, Steinheim, Germany). The tissue samples were paraffin-embedded and then 5 to

6 µm sections were cut on a Leica DSC1 rotating disk microtome (Leica Microsystems GmbH, Nussloch, Germany). Endogenous peroxidase activity was blocked with 3% H_2O_2 in phosphate-buffered saline (PBS) solution for 15 minutes. For heat-induced antigen retrieval, the slides were boiled in 0.1 mol/L citrate buffer (pH 6.0) for 15 minutes in a microwave oven at 750 W. Slides were incubated with primary antibody diluted 1:1000 in PBS solution supplemented with 5% bovine serum albumin for 15 to 18 hours (overnight) at 4°C. A streptavidin–biotin system using a biotinylated secondary antibody (donkey anti-sheep Ig; The Binding Site, Birmingham, UK), followed by incubation with a Vectastain ABC Kit (Vector Laboratories, Inc, Burlingame, CA) and diaminobenzidine (DAKOCytomation) was used as a chromogen for signal visualization. The sections were counterstained with haematoxylin. As a control for background staining, the slides were treated in the same manner, except that PBS solution was substituted for the primary and/or secondary antibody. The stained slides were examined using computer image analysis (light microscope Olympus AX70, Japan; digital camera PixeLINK PL-A642, Vitana Corp, USA; LUCIA software, version 4.71, Laboratory Imaging, Prague, Czech Republic).

Statistical Analysis

Results are presented as means ± standard error of the mean (means ± SEM) and analyzed using an unpaired Student's *t* test or by one-way analysis of variance (ANOVA) followed by a Newman–Keuls multiple range test. In all cases, a probability level of $P < .05$ was considered significant.

RESULTS

Developmental Profiles of Placental 11HSD1 and 11HSD2

The relative abundance of 11HSD1 mRNA and 11HSD2 mRNA was assessed using quantitative RT-PCR. When plotted according to the day of gestation, a dramatic decrease in 11HSD1 transcript levels was observed between days E13 and E14 followed by a smaller insignificant reduction in the following days (Figure 1). In contrast, placental 11HSD2 mRNA was more abundantly expressed during the last third of gestation and decreased from days E13 to E21, when expression was reduced to ~50%. The mean level of 11HSD2 protein decreased with advancing gestation to reach its lowest value in the last measurement before term (Figure 2).

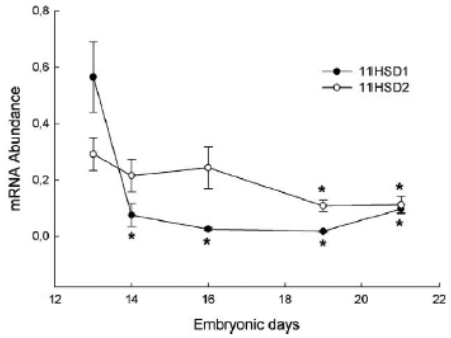

Figure 1. Gestation-related changes in placental abundance of 11HSD1 mRNA and 11HSD2 mRNA. Expression was measured using quantitative RT-PCR, and results are given as relative abundance compared with that of β-actin. Data are means ± SEM of 5 to 7 placentae in each group. *Significantly different from younger stages ($P < .05$).

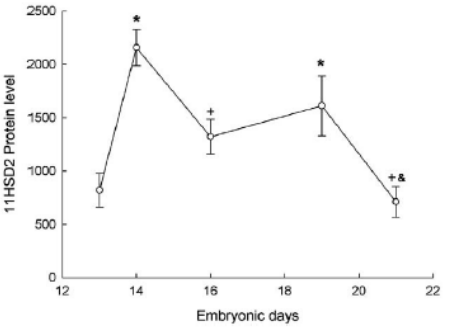

Figure 2. Gestation-related changes in placental 11HSD2 protein levels. Relative protein levels were measured by Western blot and computer-assisted densitometry based on chemiluminescence detection. Data are means ± SEM of 5 to 9 placentae in each group. *Significantly different from E13, +from E14 and &from E19 ($P < .05$).

To assess whether these changes in 11HSD1 and 11HSD2 mRNAs are associated with changes in enzyme activities, the conversion of [^{3}H]corticosterone to [^{3}H]11-dehydrocorticosterone and vice versa was measured in the presence of their respective cosubstrates. Placental 11β-hydroxysteroid dehydrogenase displayed an increase in NAD^+-dependent 11β-oxidase activity but a decrease in $NADP^+$-dependent 11β-oxidase activity, respectively

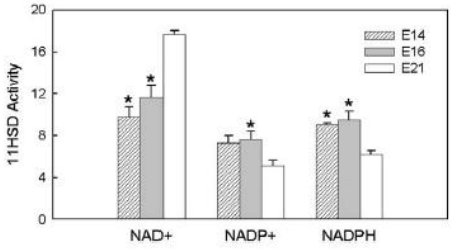

Figure 3. Oxidation of [^{3}H]corticosterone (NAD$^+$ and NADP$^+$) and reduction of [^{3}H]11-dehydrocorticosterone (NADPH) in placental homogenates on days E14, E16, and E21 of gestation. The enzymatic activity is expressed as picomoles of corticosterone or 11-dehydrocorticosterone per hour and milligrams of protein and the values are means ± SEM for 10 to 16 placentae in each group. *Significantly different from E21 ($P < .05$).

(Figure 3). In the presence of NADP$^+$, the placental 11β-oxidase activity had a similar value to the NADPH-dependent 11-reductase activity and both activities showed a similar developmental profile during gestation. In all assays, HPLC analysis showed only two peaks corresponding to corticosterone and 11-dehydrocorticosterone and no other metabolites were identified (not shown). These results indicate that the placenta is bidirectional and its ability to inactivate corticosterone is more obvious immediately before term than at the beginning of the last third of gestation.

The immunoreactivity of 11HSD2 was markedly detected in both the junctional and labyrinth zone of rat placenta throughout gestation. Figure 4A and B shows that in the labyrinth zone, the most intense staining was found in the cytoplasm of the differentiated syncytiotrophoblast cells on day E13. The fetal erythroblasts were also positive for 11HSD2. In the junctional zone, weak 11HSD2 positivity was found in the spongiotrophoblast and in the giant cells. On day E16 (Figure 4C and D), the differentiated syncytiotrophoblast of the labyrinth zone was well established. 11HSD2 was found in syncytiotrophoblast I along the interhemal membrane between the maternal space and fetal capillaries. Lower positivity was found in the cytoplasm of the spongiotrophoblast and trophoblast giant cells of the junctional zone. The mature placenta with completely established components of the labyrinth is shown on E19 (Figure 4E) and E21 (Figure 4F). 11β-HSD2 positivity was found in the syncytiotrophoblast I.

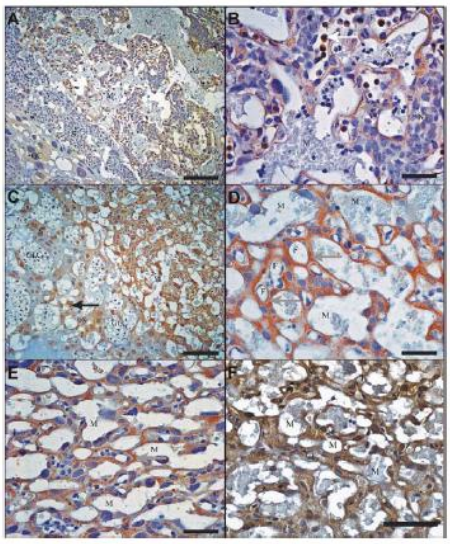

Figure 4. Immunohistochemical localization of 11HSD2 on days E13 (A, B), E16 (C, D), E19 (E), and E21 (F) of gestation. Bars: A and C, 150 μm; B, D, and E, 75 μm; F, 100 μm. M, maternal lacunae; F, fetal capillaries; GLC, glycogen cells; S, spongiotrophoblast; GC, giant cells; NT, nondifferentiated trophoblast. Black arrow, 11HSD2 in spongiotrophoblast; gray arrows, 11HSD2 in syncytiotrophoblast; white arrow, erythroblasts.

Effect of DTT on Placental 11HSD2

A comparison of the developmental profiles of 11HSD2 mRNA (Figure 1), protein (Figure 2) and NAD$^+$-dependent 11β-oxidase activity (Figure 3) indicated that 11HSD2 might be subjected to regulation at a posttranslational level. To obtain further insight into the posttranslational regulation of 11HSD2, we studied the effect of DTT on 11HSD2 activity, because dimerization has recently been shown to bring about 11HSD2 inactivation.[25,26] As shown in Figure 5, DTT stimulated 11HSD2 activity and this effect was concentration-dependent. In addition, placental 11HSD2 was more sensitive to this reducing agent immediately before term than in younger stages of gestation.

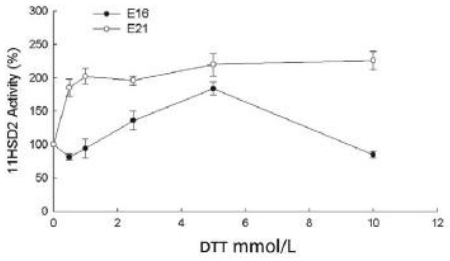

Figure 5. Effect of dithiothreitol (DTT) on 11HSD2 activity in placental microsomes on days E16 and E21 of gestation. The activity of 11HSD2 was measured as NAD^+-dependent conversion of [^{3}H]corticosterone to [^{3}H]11-dehydrocorticosterone. Values are means ± SEM of 3 experiments.

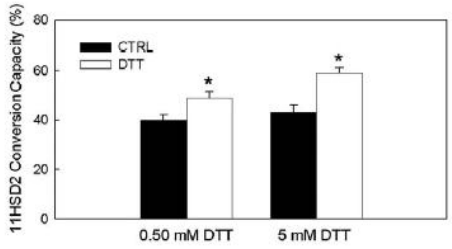

Figure 6. Effect of dithiothreitol (DTT) on 11HSD2 conversion capacity of perfused rat placenta on day 21 of gestation. Corticosterone was infused to the placenta from the maternal side at a concentration of 50 nmol/L. After 10 minutes, DTT was added to the maternal reservoir to reach a concentration of 0.5 or 5 mmol/L. Fetal effluent was sampled and analyzed for corticosterone and 11-dehydrocorticosterone and the conversion capacity was calculated as described in the section Materials and Methods. Data are given as means ± SEM of 6 experiments. *Significantly different from control period ($P < .05$).

To test the possibility that disulfide bonds are able to modulate 11HSD2 activity in intact organs, we performed experiments with isolated placentae. The placenta was perfused with corticosterone (50 nmol/L) without and subsequently with DTT (0.5 or 5 mmol/L) and the placental 11HSD2 conversion capacity was estimated. In this study, DTT at concentrations of 0.5 and 5 mmol/L caused an increase in 11HSD2 conversion capacity by 21.8% and 22.2%, respectively (Figure 6).

Effect of Gestation on the Distribution of [^{3}H]Corticosterone to the Fetus and the Activity of 11HSDs in Fetal Tissue

To determine the transplacental transfer of corticosterone and its metabolism in the fetoplacental unit, we measured the local concentration of radioactivity in maternal plasma and the fetus during steady-state infusion of [^{3}H]corticosterone. This steady-state plasma concentration was reached on the 30th minute after the initiation of infusion into the dam, lasted at least 30 minutes, and ~90% of this maternal plasma radioactivity remained in the form of [^{3}H]corticosterone. Tritiated 11-dehydrocorticosterone represented approximately 9% to 10% of the labeled corticosterone (Figure 7B) and the ratio of [^{3}H]corticosterone to [^{3}H]11-dehydrocorticosterone in maternal plasma did not change during gestation. However, in the fetuses, the ratio of tritiated corticosterone to 11-dehydrocorticosterone was significantly lower than in maternal plasma and increased with gestational age (Figure 7A; E16, 62/38; E21, 81/19).

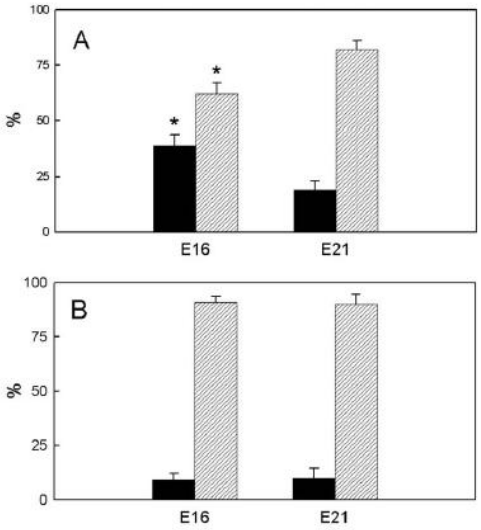

Figure 7. Percentage of [^{3}H]corticosterone (hatched columns) and [^{3}H]11-dehydrocorticosterone (black columns) in fetus (A) and maternal plasma (B) expressed as a percentage of total corticosterone and 11-dehydrocorticosterone radioactivity at steady state. Values are means ± SEM (3 to 4 animals per group). *$P < .05$ compared with value at E21.

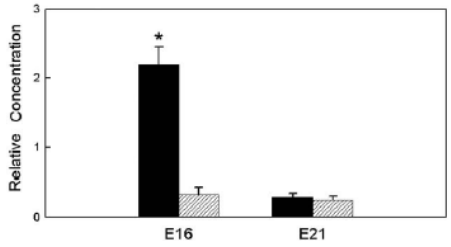

Figure 8. Effect of gestation on the steady-state concentration of [^{3}H]corticosterone (hatched columns) and [^{3}H]11-dehydrocorticosterone (black columns) in the fetus during infusion of tritiated corticosterone. Relative tissue concentration was calculated as disintegrations per gram (DPM/g) fetal tissue divided by DPM per milliliter (DPM/mL) of maternal plasma. Values represent the mean ± SEM of 9 fetuses per group. *$P < .05$ compared with value at E21.

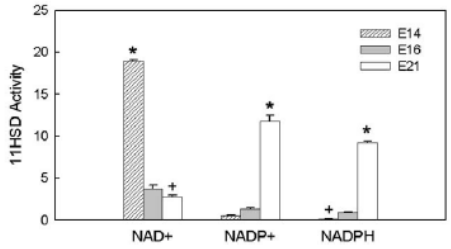

Figure 9. Oxidation of [^{3}H]corticosterone (NAD$^+$ and NADP$^+$) and reduction of [^{3}H]11-dehydrocorticosterone (NADPH) in homogenates of fetuses on days E14, E16, and E21 of pregnancy. The enzymatic activity is expressed as picomoles of corticosterone or 11-dehydrocorticosterone per hour and milligram of protein and the values are means ± SEM of 15 to 19 fetuses per group. *Significantly higher than in other ages and $^+$significantly different from E16 ($P < .05$).

The steady-state fetal concentration of [^{3}H]11-dehydrocorticosterone relative to that of maternal plasma was significantly higher on day E16 than before term (Figure 8) and this developmental pattern corresponded to that of fetal and was opposite to that of placental 11HSD2 activities (Figures 3 and 9).

DISCUSSION

Fetal development is associated with a markedly different fetal concentration of biologically active glucocorticoids, cortisol, and corticosterone, than is in the maternal blood. In human and other mammals this maternal concentration is several times higher than the corresponding value in the fetus.[8,27] In the middle stage of gestation, the rat fetal plasma concentration of corticosterone reaches a value approximately 100 times lower than the maternal plasma and even though the fetal concentration increases during the final third of gestation, its level is always lower than the corresponding maternal one.[22,28] The reason these relatively low levels are maintained is in part due to the absence of a functional fetal hypothalamic-pituitary-adrenal axis that starts to operate after E16,[29,30] and in part because of the transplacental barrier to the transfer of maternal corticosterone to fetuses together with the corticosterone metabolism in fetoplacental unit. As we have shown recently, this barrier in rats is not complete[6] and the dams of rat contribute to the fetal corticosterone concentration to a similar extent as was shown for cortisol in other mammals.[8,31]

This study confirms and extends the previous observations by others that placental tissue is able to convert biologically active glucocorticoids to their inactive derivatives and vice versa and that this metabolic capacity undergoes developmental changes. 11HSD1 has been shown to progressively increase in baboon and porcine placenta[14,21] but to decrease in rats.[32] Similarly, 11HSD2 increases during gestation in humans, baboons, and swine[14,21,33] but decreases in mice.[19,20] The rat data remain controversial.[28,32,34] This discrepancy might result from the various methods used to estimate the developmental profiles of both enzymes (mRNA, protein level, enzyme activity in homogenates or in intact tissue slices) and from the fact that the previous papers were not able to reliably estimate 11-reductase activity as a measure of 11HSD1 in placental homogenates.[18,32] This study demonstrates that both mRNA and the protein level of 11HSD2 decrease with advancing gestation and reach their lowest values in the days immediately before term. However, these results are inconsistent with the discovery of 11HSD2 activity in placental homogenate. This activity increased during gestation in a similar way to the ability of placental tissue slices to convert corticosterone to 11-dehydrocorticosterone.[32] Whereas 11HSD2 is unable to use NADP$^+$ as a cosubstrate, 11HSD1 is able to bind and use both NAD$^+$ as NADP$^+$ (preferential substrate)[35] such that NAD$^+$-dependent 11β-oxidation reflects both 11HSD2 and 11HSD1. However, 2 findings indicate that the activity of placental 11HSD2 is upregulated during gestation. First, NADP$^+$-dependent 11β-oxidase activity

is nearly identical to NADPH-dependent 11-reductase activity whereas NAD^+-dependent 11β-oxidase activity is significantly higher. Second, the developmental patterns of NAD^+-dependent 11β-oxidase activity is opposite to those of $NADP^+$-dependent 11β-oxidase and NADPH-dependent 11-reductase. The NAD^+-dependent activity increases whereas $NADP^+$-dependent and NADPH-dependent activities decrease during gestation. The data presented here thus support the possibility that, in addition to the genomic regulation of 11HSD2, there is also a posttranslational regulation of this enzyme. Such regulation has been proposed by the formation of inactive dimers that act as a latent form of 11HSD2.[25,26,36] The results of this study indicate that a similar mechanism may also operate in placenta and thus a regulated, reversible dimerization and monomerization of 11HSD2 molecules could serve as a mechanism of rapid modulation of placental enzymatic activity. However, the finding that DTT more effectively increased enzyme activity at E21 than at earlier stages, suggests that dimerization was higher at E21 than at E16, yet activity at E21 was relatively high compared with protein level at this time. Additionally, prostaglandins have been recently shown to attenuate 11HSD2 activity without any alteration of 11HSD2 mRNA in JEG-3 cells, a syncytiotrophoblast-like cell line derived from human choriocarcinoma.[37] Taken together, these data indicate that stimulation of placental 11HSD2 at the end of gestation is mediated by a novel mechanism that does not involve monomerization of 11HSD2.

The coexpression of 11HSD2 and 11HSD1 in the placenta suggests that 11HSD1 might also modulate the transplacental transfer of corticosterone. If we accept the general consensus that 11HSD1 acts as an 11-reductase converting 11-dehydrocorticosterone to corticosterone, we cannot exclude the possibility that placental 11HSD1 might contribute to the placental delivery of maternal glucocorticoids to the fetus. Because 11-oxo derivatives of glucocorticoid hormones are not bound to plasma binding proteins there is abundant free 11-dehydrocorticosterone at a tens of nanomolar concentration in maternal blood[38] that could be a substrate for placental 11HSD1 during transfer of maternal 11-dehydrocorticosterone to fetal circulation. Nevertheless, the experiments with perfused human placenta showed that the percentage of cortisol formation from cortisone is much less than the formation from the same dose of cortisol infusion or that 11-reductase activity is completely absent.[4,5]

Our study provides direct evidence that glucocorticoids of maternal origin are able to access the fetal compartment and corticosterone either partially escapes its inactivation to 11-dehydrocorticosterone in the fetoplacental unit or 11-dehydrocorticosterone is reactivated to corticosterone via fetal 11HSD1. However, the activity of fetal 11HSD1 is very low in E16 whereas 11HSD2 is relatively high. Thus, it is reasonable to consider that some maternal corticosterone in younger stages of placenta escapes its inactivation. Even if the amount of glucocorticoids transferred across the placenta is generally low,[8] maternal glucocorticoids that transverse the placenta before the activation of ACTH secretion and maturation of the adrenal corticosteroid metabolism seem to be the only glucocorticoid hormones in the fetal compartment. In rats, it has been found that fetal adrenocorticotropic activity begins to secrete ACTH on days E17 and E18, which is also when the fetal adrenal gland gains the ability to synthesise corticosteroids.[29,30] Thus, it is reasonable to consider that the corticosterone in the rat fetus before E17 is of maternal origin. Based on analysis of the extractable radioactivity, it is obvious that the ratio of corticosterone to 11-dehydrocorticosterone in fetuses on E16 is much smaller that in maternal plasma and also smaller than this ratio on E21. Hence, the fetoplacental unit in earlier stages of gestation appears to provide a greater capacity to inactivate maternal corticosterone. The upregulation of corticosterone oxidation in the earlier stages of gestation is consistent with the increased steady-state concentration of [^{3}H]11-dehydrocorticosterone in the fetus relative to that in maternal plasma. If we consider the silencing of 11HSD1 and considerable 11HSD2 activity in the fetus during earlier stages of gestation and much lower activity of fetal 11HSD2 at the end of gestation together with the upregulation of 11HSD1 activity, these findings suggest that fetal 11HSD2 plays an important role in excluding maternal corticosterone from fetal tissue in the earlier stages of gestation. In agreement with this, Brown et al[19] found a widespread abundance of 11HSD2 mRNA in mouse embryo from E9 to E12 that dropped to low or undetectable levels between E12.5 and E13.5 (term E19).

There seems to be a different situation at the end of gestation, when endogenous corticosterone acts as a powerful morphogenetic factor, influencing the development of a range of processes in the fetus.[1] Fetal 11HSD2 is dramatically decreased and 11HSD1 increased (Figure 9), primarily due to changes in the lung and liver,[39-41] and in

addition, endogenous corticosterone production by the fetal adrenal gland is increased.[29] The concentration of fetal [^{3}H]11-dehydrocorticosterone was significantly lower in E21 fetuses than in E16 fetuses, which is consistent with the decreased 11HSD2 and increased 11HSD1 activities in fetuses between E16 and E21. Thus, the fetus instead of placenta seems to have a more important role in glucocorticoid metabolism in fetoplacental unit at the end gestation

In summary, this study demonstrates the developmental changes in placental and fetal 11HSD1 and 11HSD2 in the last third of gestation and suggests that both the placental barrier and the oxidation of corticosterone within the fetoplacental unit are able to protect the fetus against maternal glucocorticoids. This protective function seems to play a more important role at the beginning of the last third of gestation than immediately before term, and both placental and fetal 11β-oxidation of glucocorticoids is involved in this process. Moreover, this study suggests that in addition to transcriptional regulations, placental 11HSD2 activity may be regulated in vivo by dimer–monomer formation. Collectively, these data emphasize, how the developmental alterations of glucocorticoid metabolism in placenta and fetus may influence the fetal concentration of biologically active glucocorticoids. Further studies will be required to determine the direction of 11HSD1 reaction in placental and fetal tissues in vivo. Recent findings show that the activity of 11HSD1 switches from an oxidase to a reductase upon coexpression with the enzyme hexose-6-phosphate dehydrogenase that is thought to modulate the local concentration of nucleotide cosubstrates and is closely associated with 11HSD1 in the membrane of endoplasmic reticulum.[42,43]

ACKNOWLEDGMENTS

We thank Mrs I. Mezteková and I. Muricová for technical assistance.

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IV.

DEXAMETHASONE AND BETAMETHASONE ADMINISTRATION DURING PREGNANCY AFFECTS EXPRESSION AND FUNCTION OF 11beta-HYDROXYSTEROID DEHYDROGENASE TYPE 2 IN THE RAT PLACENTA

Reproductive Toxicology 28 (2009) 46–51

Contents lists available at ScienceDirect

Reproductive Toxicology

journal homepage: www.elsevier.com/locate/reprotox

Dexamethasone and betamethasone administration during pregnancy affects expression and function of 11β-hydroxysteroid dehydrogenase type 2 in the rat placenta

Zuzana Vackova[a,c], Karla Vagnerova[b], Antonin Libra[c], Ivan Miksik[b], Jiri Pacha[b], Frantisek Staud[a,*]

[a] Department of Pharmacology and Toxicology, Faculty of Pharmacy in Hradec Kralove, Charles University in Prague, Hradec Kralove, Czech Republic
[b] Institute of Physiology, Czech Academy of Sciences, Prague, Czech Republic
[c] Genert Biotech s.r.o., Hradec Kralove, Czech Republic

ARTICLE INFO

Article history:
Received 4 September 2008
Received in revised form 11 February 2009
Accepted 13 February 2009
Available online 24 February 2009

Keywords:
11β-Hydroxysteroid dehydrogenase
Metabolism
Rat placenta
Dexamethasone
Betamethasone

ABSTRACT

Placental 11β-hydroxysteroid dehydrogenase type 2 (11β-HSD2) is the key enzyme which protects the fetus from overexposure to glucocorticoids (GCs) by their oxidation into inactive derivates. Several recent studies suggest that 11β-HSD2 expression is subjected to regulation by antenatal steroid therapy. In our study we investigated the effect of two commonly used synthetic steroids, dexamethasone (DXM) and betamethasone (BTM), on the expression and function of 11β-HSD2 in the rat placenta. Pregnant rats were pretreated with low (0.2 mg/kg) or high (5 mg/kg and 11.5 mg/kg for DXM and BTM, respectively) i.m. doses of GCs. 11β-HSD2 expression was investigated using real-time RT-PCR and Western blotting; conversion capacity of 11β-HSD2 was assessed by dual perfusion of the rat placenta. Significant increase in placental 11β-HSD2 mRNA expression was found in rats treated with DXM, however, this alteration was not observed on protein level. BTM had no effect on either mRNA or protein levels of 11β-HSD2. Functional studies revealed that both GCs significantly reduced the metabolism of corticosterone by the placenta. Our data indicate that placental barrier function mediated by 11β-HSD2 might be considerably impaired by the antenatal therapy with DXM and BTM. In addition, the discrepancy between expressional and functional studies suggests that sole analysis of expressional changes of 11β-HSD2 at mRNA and/or protein levels cannot convincingly predict the role of GC treatment on 11β-HSD2 function in the placental barrier.

1. Introduction

It is well documented that the intrauterine environment and specific factors acting during critical developing periods determine both the neonatal and adult well-being. In particular, prenatal exposure to endogenous glucocorticoids (GCs) is in the centre of interest since GCs are essential for the maturation of fetal organs, growth and preparing the fetus for birth [1–3]. Although GCs are highly lipophilic and can readily cross the placenta, during the whole course of pregnancy the levels of circulating corticosteroids in the fetus are considerably lower than those in the mother. This gradient is maintained by the activity of placental 11β-hydroxysteroid dehydrogenase (11β-HSD), the enzyme responsible for the interconversion of cortisol and corticosterone and their inactive 11-keto products cortisone and 11-dehydrocorticosterone, respectively [4].

Two types of 11β-HSD are now distinguished. Type 1 (11β-HSD1) is a low affinity NADP(H) preferring enzyme which appears to act predominantly as a reductase with high K_m in GC target tissues [5]. Conversely, type 2 (11β-HSD2) is a NAD^+-dependent oxidase with K_m in the nanomolar range for cortisol and corticosterone which catalyses their unidirectional conversion to inactive 11-oxo metabolites [6,7]. Although the presence of both types has been demonstrated in the placenta [8–10], it is generally accepted that the predominant form limiting the materno-fetal passage of GCs is 11β-HSD2 with its oxidative activity. 11β-HSD2 has been localized to the syncytiotrophoblast layer of the placenta, the main site of materno-fetal exchange [8,11]. It forms an active component of the placental barrier with the potency to protect the fetus against GC overexposure. Attenuated expression and activity of 11β-HSD2 allowing enhanced transplacental passage of GCs has been linked to the intrauterine growth restriction and increased occurrence of cardiovascular and metabolic diseases in adult life (for detailed review see [12]).

The expression and activity of placental 11β-HSD2 is not uniform across gestation. The variation most likely reflects tissue-specific regulation of 11β-HSD2 by diverse endogenous agents

* Corresponding author at: Faculty of Pharmacy, Heyrovskeho 1203, 500 05 Hradec Kralove, Czech Republic. Tel.: +420 495067218; fax: +420 495714373.
E-mail address: frantisek.staud@faf.cuni.cz (F. Staud).

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doi:10.1016/j.reprotox.2009.02.006

Table 1
Sequences of primers used in real-time RT-PCR.

Gene	NCBI sequence	Sequence 5′ → 3′	Product length (bp)	Localization
rβ-2-microglobulin	NM_012512	TGC CAT TCA GAA AAC TCC CCA (f) TAC ATG TCT CGG TCC CAG GTG A (r)	303	64–366
r11β-hsd1	NM_01780	GGCTTCATAGACACAGAAACAG (f) TTCCCAAGCAGAAGTGGAG (r)	161	721–881
r11β-hsd2	NM_017081	CAG CAG GAG ACA TGC CAT AC (f) CAC ATT AGT CAC TGC CTC TGT C (r)	161	785–945

[13–15]. Moreover, the changes in 11β-HSD2 activity and expression throughout pregnancy express marked variability among species. Increasing activity of placental 11β-HSD2 with advancing pregnancy was demonstrated in the human, baboon and rat placenta [16–18]. However progressive increase in 11β-HSD2 expression with advanced pregnancy was demonstrated only in human [19,20] and baboon [17] placenta while an opposite trend was demonstrated in the rat [21,22]. Furthermore, it has been recently suggested that 11β-HSD2 is subjected to regulation by various exogenous agents; particularly, by synthetic GCs. However, the results of recent studies remain controversial. Clarke et al. found that administration of cortisol to pregnant ewes reduced placental 11β-HSD2 activity [23]. Likewise, prenatal exposure to dexamethasone (DXM) was followed by decreased 11β-HSD2 expression in the ovine placenta during mid-gestation [24]. Contrary to these observations, betamethasone (BTM) and DXM treatment was demonstrated to stimulate the expression of 11β-HSD2 in baboon placenta [25] and expression and activity of 11β-HSD2 in primary culture of human trophoblast [26], respectively.

In the present study we have investigated the effect of synthetic GCs on 11β-HSD2 expression and function in the rat placenta. In particular, we have focused on DXM and BTM since these steroids are commonly administered to pregnant women in managing premature delivery [27]. Changes in the expression of 11β-HSD2 on mRNA and protein levels were investigated using quantitative real-time RT-PCR and Western blotting techniques. In addition, we have recently validated the *in-situ* method of perfused rat placenta as a viable model to study placental steroid metabolism by 11β-HSD2 [21]. Using this method we were able to examine the effect of antenatal administration of GC on functional capacity of placental 11β-HSD2 to metabolize corticosterone at term.

2. Materials and methods

2.1. Animals

All experiments were approved by the Ethical Committee of the Faculty of Pharmacy (Hradec Kralove, Charles University in Prague) and were carried out in accordance with the Guide for the Care and Use of Laboratory Animals, 1996 and the European Convention for the protection of vertebrate animals used for experimental and other scientific purposes, Strasbourg, 1986. Pregnant Wistar rats were purchased from Biotest Ltd. (Konarovice, Czech Republic) and were maintained in 12/12-h day/night standard conditions with water and pellets ad libitum. A group of 20 dams was pretreated with five doses of DXM ($n = 9$) (Medochemie Ltd., Limassol, Cyprus) or BTM ($n = 11$) (Schering-Plough, Herouville, France) administered intramuscularly into the hindlimb muscles in 24-h intervals (regularly at 9:00 a.m.) at 16–20 gestation days. This treatment regime was chosen to investigate the effect of GC treatment in the third term of pregnancy and with respect to previously published studies [28,29]. Two different doses of each GC were applied: low doses (0.2 mg/kg of DXM; $n = 5$ and BTM; $n = 6$) or high doses (5 mg/kg and 11.5 mg/kg of DXM; $n = 4$ and BTM; $n = 5$, respectively). The administered volume was 0.5 ml. A control group of five dams received vehicle treatment (0.9% NaCl). Experiments were performed on day 21 of gestation. Fasted rats were anesthetized with pentobarbital (Nembutal, Abbott Laboratories, North Chicago, IL) in a dose of 40 mg/kg administered into the tail vein and dual perfusion of the rat placenta was performed. At the end of the experiment the perfused placenta along with four randomly selected placentas, were dissected free of maternal tissues and fetal membranes and stored at −70 °C until analysis.

2.2. Real-time RT-PCR

Total RNA from frozen tissue samples was isolated using TriReagent (Molecular research centre, Inc., Cinncinati, USA). The RNA concentrations were determined from the A_{260} measurement. Purity and integrity of RNA was confirmed by calculation of the UV absorbance ratio A_{260}/A_{280} and electrophoresis on 2.5% agarose gel.

Before the initiation of the real-time RT-PCR analysis, stability of the following genes was compared: *β-2-microglobulin*, *Surf1*, *Nup54* and *Polr2a*. For subsequent PCR analyses, *β-2-microglobulin* gene was chosen as the least regulated one. In addition to *β-2-microglobulin* gene normalization, we have related the expression of 11β-HSD to the total amount of RNA. The results did not change significantly and there was no influence of the denominator.

Single strand cDNA was prepared from 1 μg of total RNA by reverse transcription with M-MLV transcriptase (Finnzymes Oy, Espoo, Finland) using oligo$(dT)_{15}$VN primer (Generi Biotech Ltd., Hradec Kralove, Czech Republic) and porcine RNase inhibitor (TaKaRa Bio Inc., Otsu, Shiga, Japan). Primers for amplification of segments of rat target (*11β-hsd1* and *11β-hsd2*) and housekeeping (*β-2-microglobulin*) genes were designed using the Vector NTI Suite software (Infomax, Bethesda, MD, USA). Specifications are given in Table 1. Primers for housekeeping genes *Surf1*, *Nup54* and *Polr2a* were purchased from Generi-Biotech Ltd. (Hradec Kralove, Czech Republic). Real-time PCR analysis was performed on iCycler iQ (Bio-Rad Laboratories, Inc., Hercules, CA). HotStart Taq DNA polymerase (AB gene, Epsom, UK) was employed for cDNA amplification under the following conditions: 1.5 mM $MgCl_2$, 0.2 mM dNTP, 0.025 U/μl polymerase, 0.3 μM of each primer, SYBR® Green I in 1:100,000 dilution. The temperature profile was 95 °C for 14 min and 40 repeats of cycle consisting of 95 °C for 15 s, 60 °C for 20 s and 72 °C for 20 s. Calibration curves used for the calculation of reaction effectivity were generated from amplification of dilutions of randomly selected cDNA sample, the dilution factor was 5. The amplification efficiencies varied between 95% and 105%. Amplification of the desired sequence was confirmed by melting curve analysis (T_m were 86.5 °C, 84.5 °C and 87.5 °C for *β-2-microglobulin*, *11β-hsd1* and *11β-hsd2*, respectively). PCR products were separated on 2% agarose gel in the presence of ethidium bromide, visualized under ultraviolet light and compared with low-molecular-weight ladder (25–766 bp) (New England Biolabs, Hertsfordshire, UK). The analysis of real-time amplification curves and subtraction of Ct values was performed using iCycler iQ 3.0 software (Bio-Rad Laboratories, Inc., Hercules, CA). Relative expression of 11β-HSD mRNA was calculated from the real-time RT-PCR efficiencies and the Ct deviation of a sample versus control using the method of Pfaffl [30]. Results are expressed as a percentage of control ± SEM.

2.3. Western blotting analysis

Western blots were performed as described previously [21]. Briefly, placenta and kidney as a positive control were homogenized, centrifuged at 400 × *g* for 10 min and the supernatant centrifuged again at 100,000 × *g* for 60 min. The pellet was resuspended and sonicated in Laemmli buffer containing β-mercaptoethanol (2%), boiled for 3 min and stored at −20 °C until analysis.

Proteins were separated by 10% SDS-PAGE gel electrophoresis and electroblotted in a semi-dry blotting system. The blots were incubated with a sheep anti-rat 11β-HSD2 polyclonal antibody (Chemicon International, Inc., Temecula, CA, USA) and subsequently incubated with peroxidase-conjugated rabbit anti-sheep immunoglobulin G (Pierce Biotechnology, Inc., Rockford, IL, USA). The protein bands were visualized using Super Signal West Femto substrate (Pierce Biotechnology, Inc., Rockford, IL, USA) and detected by luminescence analyzing system LAS-1000 (Fuji, Tokyo, Japan).

For quantification, equal amounts of protein from each sample (13 μg of total protein) were used and the chemiluminescent signal of the measured immunoreactivity was in the linear range in terms of protein amounts used in this analysis. The results were calculated as a ratio of chemiluminescent signal to mg of protein ± SEM. Four samples from each group were analyzed.

2.4. Dual perfusion of the rat placenta

The method of dually perfused rat placenta was used as described previously [21]. Briefly, one uterine horn was excised and allowed to dive in the heated Ringer saline (37 °C). A catheter was inserted into the uterine artery proximal to the blood

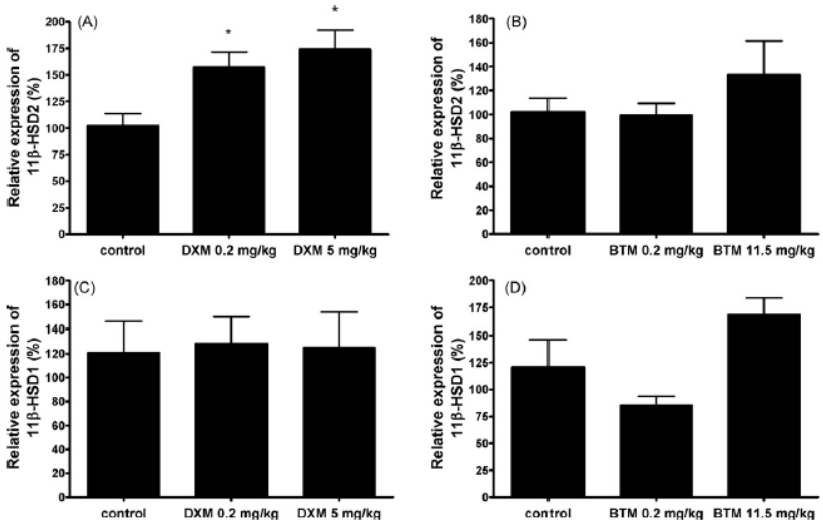

Fig. 1. Real-time RT-PCR analysis of mRNA expression of 11β-HSD2 (A and B) and 11β-HSD1 (C and D) in the rat placenta. Pregnant rats were pretreated with DXM (0.2 mg/kg or 5 mg/kg), $n = 9$ or BTM (0.2 mg/kg or 11.5 mg/kg), $n = 11$. Relative expression was calculated by using the method of Pfaffl [30] and is expressed as mean percentage of control ± SEM; $^*p < 0.05$.

vessel supplying a selected placenta and connected with the peristaltic pump. Krebs' perfusion liquid (exposed to the mixture of 95% oxygen and 5% carbon dioxide) containing 1% dextran was brought from the maternal reservoir at a rate of 1 ml/min. The uterine vein, including the anastomoses to other fetuses, was ligated behind the perfused placenta and cut so that maternal solution could leave the perfused placenta. The selected fetus was separated from the neighboring ones by ligatures. The umbilical artery was catheterized using 24-gauge catheter and connected with the tubing by which the fetal perfusion liquid from the fetal reservoir was supplied at a rate of 0.5 ml/min. The umbilical vein was catheterized in a similar manner and the selected fetus was removed. Before the start of each experiment, the fetal vein effluent was collected into pre-weighted glass vials to check a possible leakage of perfusion solutions from the placenta. In the case of leakage, the experiment was terminated. Maternal and fetal perfusion pressures were monitored continuously throughout the perfusion experiments.

Immediately after successful surgery maternal side of the placenta was perfused with either 50 nM or 200 nM concentration of corticosterone with [^{3}H]corticosterone as a tracer. After 10 min stabilization period sample collection started. Fetal effluent samples were collected for 30 min of perfusion and the steroids extracted using Sep-Pak cartridges. The analysis of steroids was performed by HPLC using an Agilent 1100 system (Agilent, Palo Alto, CA, USA) and radioactivity detector (Radiomatic 150TR, Canberra Packard, Meriden, CT, USA) with a flow cell. Corticosterone and its reaction products were detected according to a previously described procedure [31]. Conversion capacity of 11β-HSD2 was calculated as a ratio of 11-dehydrocorticosterone (metabolite) concentration to the concentration of total corticosteroids (corticosterone and 11-dehydrocorticosterone) and presented as means ± SEM.

2.5. Statistical analysis

Data from real-time RT-PCR were analyzed using the REST©software [32] to assess statistical significance. Results of Western blot analyses and dual perfusion experiments were analyzed by ANOVA followed by Bonferronils comparison test. Differences of $p < 0.05$ were considered statistically significant.

3. Results

3.1. Effect of GC pretreatment on birth weights

Decline in fetal weights was observed in rats treated with GCs when compared to control group, however, statistical significance was found only in high dose DXM group of rats ($p < 0.001$). Means of fetal weights were 3.52 ± 0.54 g, 3.23 ± 0.36 g, 1.75 ± 0.3 g, 3.48 ± 0.18 g and 3.05 ± 0.31 g for control, 0.2 mg/kg DXM, 5 mg/kg DXM, 0.2 mg/kg BTM and 11.5 mg/kg BTM, respectively. No significant changes in placental weights were observed.

3.2. Effect of GC pretreatment on mRNA and protein levels of 11β-HSD in the rat placenta

Real-time RT-PCR analysis demonstrated the presence of both 11β-HSD1 and 11β-HSD2 mRNA in the rat placenta. Rat liver and kidney were used as positive controls (data not shown). Quantitative data evaluation revealed a distinct enhancement of placental 11β-HSD2 mRNA in dams pretreated with DXM (Fig. 1A). Both dosage regimens lead to statistically significant enhancement in 11β-HSD2 mRNA expression. The mRNA expression was increased up to 57.1% and 74% at 0.2 mg/kg DXM and 5 mg/kg DXM, respectively. In contrast, antenatal treatment of rats with either 0.2 mg/kg or 11.5 mg/kg of BTM had no effect on placental 11β-HSD2 mRNA expression (Fig. 1B). In the case of 11β-HSD1, neither DXM nor BTM administration affected the expression at mRNA level (Fig. 1C and D).

The immunoreactive signal of 11β-HSD2 was localized to microsomal fractions and was detected as a major band of 40 kD both in the kidney and placenta. Contrary to the real-time RT-PCR data, Western blotting analysis of placentas obtained from pregnant rats treated with high doses of DXM did not reveal any changes in placental protein content. Similarly, no alterations of placental 11β-HSD2 protein expression were observed in animals treated with high doses of BTM (Fig. 2).

3.3. Effect of GC pretreatment on corticosterone metabolism in perfused rat placenta

In perfusion studies corticosterone was used as a substrate of 11β-HSD2. Conversion capacity of placental 11β-HSD2 was investigated at two corticosterone concentrations: 50 nM

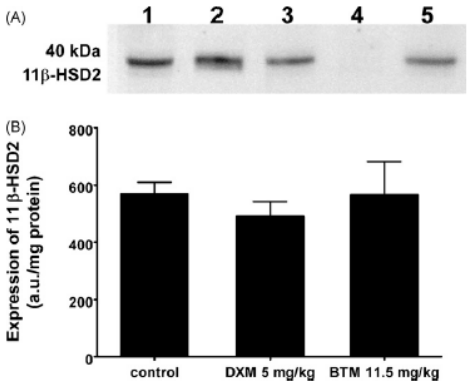

Fig. 2. Western blotting analysis of 11β-HSD2 expression in the rat placenta. (A) Representative Western blot of four similar analyses: (1) rat placenta – control (2) rat kidney, (3) rat placenta – pretreatment with BTM 11.5 mg/ml, (4) cytosolar fraction – negative control, (5) rat placenta – pretreatment with DXM 5 mg/kg. Protein load: 13 μg of total protein. (B) Expression of 11β-HSD2 calculated as a ratio of chemiluminescent signal to mg of protein. Results are expressed as mean values ± SEM, $n = 4$.

(reflecting physiological concentrations [33]) and 200 nM (supra-physiological concentrations). 11β-HSD2 conversion capacity was influenced by both substrate concentration and GC pretreatment. As in our previously published study [21], significant reduction in 11β-HSD2 conversion capacity due to enzyme saturation was observed at supra-physiological corticosterone concentration inflow (conversion capacity – 40.2 ± 8.3% and 16.3 ± 4.3% at 50 nM and 200 nM corticosterone, respectively) (Fig. 3).

Pretreatment with DXM reduced 11β-HSD2 conversion capacity in a dose dependent manner (conversion capacity – 30.2 ± 4% and 17.2 ± 3.2% at 0.2 mg/kg DXM and 5 mg/kg DXM, respectively) (Fig. 3A). In BTM treated group of rats low doses did not affect the conversion capacity of 11β-HSD2, however, high doses of BTM lead to significant reduction in corticosterone metabolism (conversion capacity – 40.9 ± 2.8% and 15.3 ± 4.9% at 0.2 mg/kg and 11.5 mg/kg BTM, respectively).

Similar pattern of changes in placental steroid metabolism caused by GC administration was found at 200 nM corticosterone inflow concentration (Fig. 3B). However, the fall in conversion capacity of 11β-HSD2 was statistically significant only in rats pretreated with high doses of DXM (conversion capacity – 4.3 ± 1.6%).

4. Discussion

Preservation of normal activity and expression of 11β-hydroxysteroid dehydrogenase type 2 (11β-HSD2) in the placenta is very important in the view of healthy development of a new individual [34]. 11β-HSD2 forms an active component of the placental barrier which controls the transplacental passage of glucocorticoids protecting the fetus against excessive GC exposure [35]. It has been demonstrated that 11β-HSD2 expression and activity is regulated by a diversity of endogenous as well as exogenous substances [36–40]. Synthetic GCs, for example, have been recently suggested to alter the expression and activity of placental 11β-HSD2 [26]. Taking into account that synthetic GCs, particularly, DXM and BTM, are often administered to pregnant women to prevent respiratory distress syndrome in premature babies [41], the elucidation of the effect of GCs on 11β-HSD2 expression and activity is strongly demanded. Several studies using variety of experimental approaches have been employed to clarify the effect of synthetic GCs on placental 11β-HSD2 [23–25], however, no definite conclusions have been drawn up to date.

We have recently investigated the effect of DXM and BTM on 11β-HSD2 mRNA expression in the human choriocarcinoma Jeg3 cells [42]. 11β-HSD2 expression tended to decline with increasing steroid concentration and incubation period, however, this trend was not statistically significant. In the present study we followed the effect of DXM and BTM pretreatment on expression and function of 11β-HSD2 in the rat placenta. Our data show that antenatal administration of DXM significantly enhanced the mRNA expression of rat placental 11β-HSD2. This increase was apparent at both low and high doses of DXM. The mechanism by which DXM induces expression of 11β-HSD2 was not examined in our study, however, van Beek et al. proposed that this effect is caused by increased 11β-HSD2 transcription, enhanced mRNA stability and prolonged mRNA half life mediated by activated glucocorticoid receptor [26].

Unlike DXM, antenatal BTM treatment did not cause any significant changes in 11β-HSD2 mRNA level. These results suggest that DXM and BTM differ in their tissue specific effects. The species-

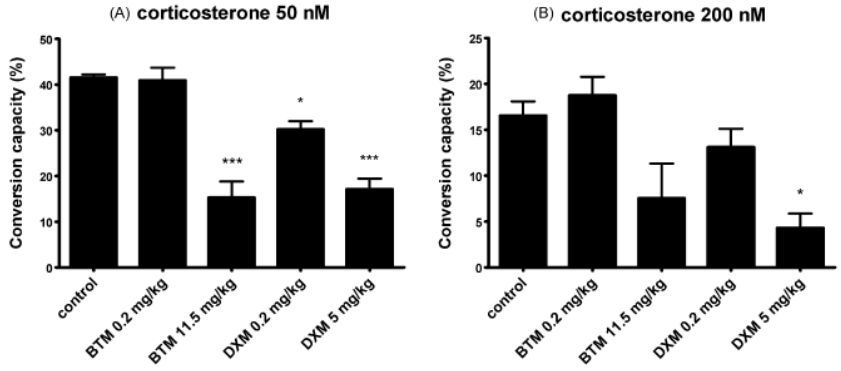

Fig. 3. The effect of GC pretreatment on 11β-HSD2 conversion capacity in the rat placenta. Corticosterone was infused into the placenta via uterine artery at a concentration of 50 nM (A) or 200 nM (B) and fetal effluent was sampled and assayed for corticosterone and 11-dehydrocorticosterone. Conversion capacity of 11β-HSD2 was calculated as a ratio of 11-dehydrocorticosterone concentration to the concentration of total corticosteroids and expressed as a percentage of mean ± SEM; $^{*}p < 0.05$, $^{***}p < 0.001$.

specific variability in transactivation response to DXM and BTM has been recently studied by Tanigawa et al. [43]. They documented that esterified BTM had only partial transactivation agonistic activity mediated by rat glucocorticoid receptor when compared with esterified DXM and non-esterified BTM and DXM. Since the injections applied to pregnant rats contain esterified DXM or BTM we speculate that the lower transactivation effect of esterified BTM may underlie the variation in gene transcription induction between DXM and BTM.

Furthermore, the effect of GCs on placental 11β-HSD2 protein content has been investigated. BTM treatment has been recently documented to stimulate 11β-HSD2 expression in the baboon placenta [25], however, in our study neither DXM nor BTM affected the expression of 11β-HSD2 in the rat placenta at the protein level. Inconsistencies between changes at mRNA and protein levels have been previously described for other placental proteins as well [44,45]. We assume that the processes involved in post-transcriptional/translational regulation may mask the stimulatory effect of DXM; nevertheless, detailed examination is needed to clarify these observations.

Although, functional studies have demonstrated that the net dehydrogenase activity in total rat placenta increases with advanced pregnancy [16], the expression of 11β-HSD2 declines as term approaches [21,22]. An opposite pattern of changes in expression was found for 11β-hydroxysteroid dehydrogenase type 1 (11β-HSD1) [8] indicating that 11β-HSD1 participates in placental GC metabolism at term in the rat. Furthermore, it has been recently reported that mRNA expression and reductase activity of placental 11β-HSD1 is stimulated by cortisol and DXM [46,47]. In our experiments, however, no changes in the expression of 11β-HSD1 at mRNA level were observed in placentas of rats pretreated with DXM or BTM. Nevertheless, since neither the protein content nor functional activity of 11β-HSD1 were investigated in our study, the overall effect of GCs treatment on 11β-HSD1 expression and activity remains to be elucidated.

The impact of synthetic GCs on placental 11β-HSD2 has been recently studied in rats [16], ovines [24], primates [25] and human trophoblast cells *in vitro* [26]. However, direct evidence of the effect of antenatal GC therapy on placental barrier function of 11β-HSD2 has not been investigated to date. This is the first report showing the influence of GCs on functional activity using perfused rat placenta. We have previously established the *in-situ* technique of dually perfused rat placenta to study the placental corticosteroid metabolism mediated by 11β-HSD2 [21]. This technique enables us to investigate the effects of maternal drug administration on the placental 11β-HSD2 steroid metabolism which is not feasible in humans. Surprisingly, our findings from *in situ* perfusion experiments did not correspond with RT-PCR and Western blotting data; on the contrary, functional studies revealed that both GCs are capable to significantly impair the steroid metabolism within the rat placenta. The administration of both doses of DXM and high doses of BTM caused decreased corticosterone inactivation, allowing higher amounts of corticosterone to reach fetal circulation. At physiological concentrations of corticosterone (50 nM) the calculated conversion capacity of 11β-HSD2 was reduced by 24.9%, 57.5% and 62% at 0.2 mg/kg DXM, 5 mg/kg DXM and 11.5 mg/kg BTM, respectively. Similar pattern of changes, although, less pronounced, was observed at 200 nM corticosterone placental inflow.

The differences between expressional and functional experiments observed in our study could be attributed to other interacting factors such as the interference of 11β-HSD1, up-regulated by GCs treatment. However, results from RT-PCR do not support this hypothesis. Furthermore, in our previous study [21], no other metabolites than 11-dehydrocorticosterone were detected in the fetal effluent. Another possible factor affecting perfusion experiments could result from the competition between corticosterone and synthetic GCs on binding sites of 11β-HSD2 which could result in decreased conversion of corticosterone. However, in our experimental setup, the selected placenta was initially washed out by perfusion solution for 10 min before the collection of samples started; subsequently no synthetic glucocorticoid was present in the perfusion liquid during the perfusion experiment. Only trace amounts of DXM or BTM could have remained in the placenta after maternal pretreatment. Nevertheless, considering their short biological half lives ($T_{1/2}$ – 2–3 h) [48,49] it is evident that 24 h after the last administered dose the level of steroids in the organism is negligible. Therefore, interference of corticosterone with DXM or BTM during perfusion studies can be ruled out. The discrepancies between expressional and functional studies thus remain to be elucidated; nevertheless, they strongly suggests that sole analysis of expressional changes of 11β-HSD2 at mRNA and/or protein levels cannot reliably predict the role of GC treatment on 11β-HSD2 function in the placental barrier. Without functional studies, results from real-time RT-PCR and Western blotting analyses could have lead to a false conclusion that DXM and BTM administration has only little impact on 11β-HSD2 function in the placenta.

In conclusion, we show the effect of synthetic GCs, DXM and BTM, on expression and function of placental 11β-HSD2 in rats. Our results reveal that only DXM but not BTM enhances 11β-HSD2 mRNA expression; however, no alterations in 11β-HSD2 protein content were detected. Despite the elevated expression observed at mRNA level, antenatal GC administration considerably decreased the metabolism of GCs in the perfused rat placenta enabling higher amounts of corticosterone to reach the fetal circulation. The impairment of corticosterone metabolism was apparent in both DXM and BTM treated groups of rats. Finally, our observations highlight the importance of functional analysis in the investigation of the effect of GCs on placental barrier.

Conflict of interest

None.

Acknowledgements

The work was supported by grant 102/2006/C/FaF from the Charles University Grant Agency and by grant KJB 5011402 from the Academy of Sciences, Czech Republic. Authors wish to thank to Anezka Kunova and Dana Souckova for their assistance in perfusion experiments.

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V.

SOUHRN

Placenta je jedinečný orgán zajišťující řadu vitálních funkcí, které jsou nezbytné pro správný průběh těhotenství a vývoj jedince. Vedle hlavní funkce přísunu živin, odvodu zplodin metabolismu a výměny plynů plní placenta také úlohu endokrinní, metabolickou a především ochrannou. Placenta je považována za jednu z fyziologických bariér organismu, která zásadním způsobem reguluje transport endogenních i exogenních látek mezi dvěma kompartmenty - krevním oběhem matky a plodu.

Do nedávné doby se předpokládalo, že placentární bariéru tvoří především buněčné vrstvy oddělující krev matky a plodu - syncytiotrofoblast a endotel fetálních kapilár. V současné době se však stále více ukazuje, že vedle této mechanické složky se na placentární bariéře podílí i složka aktivní, realizovaná činností efluxních transportérů a biotransformačních enzymů lokalizovaných ve vrstvě syncytiotrofoblastu. Efluxní transportéry jsou membránové proteiny, které aktivně (za spotřeby ATP) "pumpují" širokou škálu substrátů ven z buňky. Kinetiku transportu látek přes placentu ovlivňují zásadním způsobem především dva transportéry: P-glykoprotein (P-gp) a "breast cancer resistant protein" (BCRP). Oproti transportérům tvoří biotransformace látek v placentě pravděpodobně minoritní, avšak nezanedbatelnou složku aktivní bariéry. Jedná se zejména o enzymy cytochromu P450, konjugační enzymy II. fáze metabolismu a enzymy uplatňující se v metabolismu steroidních molekul.

V rámci této disertační práce byly studovány obě složky aktivní placentární bariéry - transportní i metabolická. Z efluxních transportérů se naše pozornost soustředila na transportér BCRP jehož expresi a aktivitu jsme studovali na modelu placenty potkana v podmínkách *in-vitro* a *in-situ*. Přítomnost BCRP jsme potvrdili v potkaní placentární buněčné linii HRP-1 i v placentě potkana na konci březosti a to jak na úrovni mRNA tak na úrovni proteinu s využitím metod real-time RT-PCR, Western blottingu a imunohistochemie. Paralelně s BCRP jsme sledovali také expresi P-gp, který byl detekován pouze v placentě potkana, zatímco v buněčné linii HRP-1 nebyl přítomen. Aktivitu BCRP v podmínkách *in-vitro* jsme potvrdili s využitím fluorescenčně značeného substrátu BCRP - BODIPY FL prazosinu a inhibitorů BCRP - GF120918 a Ko143. V souladu s výsledky expresních studií jsme v linii HRP-1 nepozorovali žádnou aktivitu P-gp. Dále jsme studovali vliv BCRP na farmakokinetiku transportu léčiv přes placentární bariéru s využitím duální perfúze placenty potkana *in-situ*. Jako modelový substrát BCRP jsme zvolili cimetidin, jehož

průchod přes placentu jsme sledovali jak v matero-fetálním tak feto-maternálním směru. Pro ověření BCRP specifického transportu byly použity inhibitory BCRP - GF120918 a fumitremorgin C. S pomocí tohoto modelu se nám podařilo prokázat, že BCRP hraje v kinetice přestupu látek přes placentu dvojí roli: 1) omezuje průchod substrátů z krve matky do plodu a 2) aktivně urychluje vylučování léčiva již přítomného v krvi plodu.

V dalších dvou studiích jsme se zaměřili na sledování exprese a aktivity enzymu 11β-hydroxysteroid dehydrogenázy (11β-HSD) jako feto-placentární bariéry průchodu glukokortikoidů (GK) z krve matky do plodu. První práce se zabývá rolí placentární 11β-HSD v metabolismu GK v průběhu březosti potkana a dále vztahem mezi aktivitou placentární a fetální 11β-HSD a jejich vlivem na poměr mezi aktivními a neaktivními formami GK v krvi plodu. Nejprve byl sledován profil exprese a aktivity placentární 11β-HSD typu 1 a 2 mezi 13. a 21. dnem březosti potkana. U obou typů placentární 11β-HSD byl pozorován pokles v expresi, avšak s odlišným profilem. Zatímco hladiny mRNA 11β-HSD1 nejprve prudce poklesly mezi 13. a 14. dnem březosti a poté zůstávaly víceméně konstantní, exprese 11β-HSD2 v poslední třetině gravidity pozvolně klesala a vždy převažovala nad 11β-HSD1. Pokles 11β-HSD2 v placentě ke konci posledního trimestru byl dále potvrzen i na úrovni proteinu. Pokles exprese 11β-HSD1 byl doprovázen také poklesem její aktivity. Oproti tomu, NAD^+ dependentní dehydrogenázová aktivita 11β-HSD2 analyzovaná v tkáňových homogenátech se ke konci gravidity zvyšovala. Tyto výsledky naznačují existenci posttranslačního regulačního mechanismu, který ovlivňuje aktivitu placentární 11β-HSD2. Ze studia aktivity 11β-HSD2 v přítomnosti dithiotreitolu *in-situ* vyplynulo, že tento mechanismus je pravděpodobně odlišný od procesu aktivace a deaktivace 11β-HSD2 reverzibilní dimerizací. Dále jsme sledovali hladiny kortikosteronu a 11-dehydrokortikosteronu v krvi plodu a porovnávali je s aktivitou placentární a plodové 11β-HSD. Z výsledků vyplývá, že na regulaci metabolismu GK a jejich hladin v krvi plodu se významně podílí aktivita plodové 11β-HSD a to především ke konci gravidity.

V následující práci jsme se zabývali vlivem prenatálně podávaných syntetických steroidů (dexametazonu a betametazonu) na expresi a konverzní kapacitu placentární 11β-HSD typu 2. Ke studiu byl opět použit model potkaní placenty. GK byly podávány březím samicím ve dvou různých dávkách (nízká nebo vysoká) v průběhu 16. - 20. dne březosti. Exprese 11β-HSD v placentách odebraných

21. den březosti byla analyzována s pomocí metod real-time RT-PCR a Western blottingem. Konverzní kapacita 11β-HSD2 byla hodnocena pomocí duální perfúze placenty potkana, jako modelový substrát pro 11β-HSD2 byl použit kortikosteron. Naše výsledky ukázaly, že ačkoliv vliv prenatální terapie syntetickými GK na expresi 11β-HSD je minimální nebo, v případě dexametazonu, omezený na transkripční úroveň, konverzní kapacita tohoto enzymu je oběma podávanými GK významně snížená. Narušení placentární bariéry tvořené 11β-HSD2 jsme zaznamenali nejen u vysokých dávek GK, ale také u nízkých dávek dexametazonu. Tato pozorování naznačují, že syntetické steroidy ovlivňují aktivitu 11β-HSD2 především na posttranslační úrovni. Naše výsledky také zdůrazňují význam funkčních studií při hodnocení vlivu exogenních látek na aktivitu enzymů placentární bariéry.

VI.
SUMMARY

Placenta is a unique organ which ensures a number of vital functions necessary for normal course of pregnancy and development of a new individual. In addition to its main function of oxygen supply and nutrient and waste product exchange, placenta also serves as an endocrine, metabolic and protective organ. Placenta is considered to be one of the physiological barriers of the organism which regulates transport of both endogenous and exogenous compounds between two compartments - maternal and fetal blood circulations.

Up to recently, the placental barrier was supposed to be formed only by cellular layers which separate maternal and fetal blood - syncytiotrophoblast and fetal capillary endothelium. However, it has been demonstrated that the activity of placental efflux transport proteins and metabolic enzymes contributes considerably to the protective function of placental barrier. Efflux transporters are membrane proteins which actively (along with consumption of ATP) "pump" a diversity of substrates out of the cell. It has been shown that the kinetics of transport of various substances across the placenta is affected predominantly by two transporters: P-glycoprotein (P-gp) and breast cancer resistance protein (BCRP). Compared to these transporters, placental biotransformation enzymes are considered to be a minor, but an important part of active placental barrier. Several placental enzymes were suggested to have an impact on the kinetics of transplacental passage of various molecules. These are in particular: cytochrome P450 enzymes, conjugation enzymes of phase II. metabolism and enzymes involved in biotransformation of steroid molecules.

In this work both components of the active placental barrier - transport and metabolic - were studied. From the group of efflux transporters we focused on BCRP transporter whose expression and activity was studied using a model of the rat placenta *in-vivo* and *in-situ*. The presence of rat BCRP mRNA and protein were confirmed in both rat placental trophoblast cell line HRP-1 and the rat placenta at the end of pregnancy. Simultaneously, we analyzed the expression of P-gp which was detected in the rat placenta but not in HRP-1 cell line. Activity of BCRP *in-vitro* was confirmed in accumulation studies with fluorescently labeled substrate of BCRP-BODIPY FL prazosine. In consistence with the results of expression studies no activity of P-gp was observed in the HRP-1 cell line. Furthermore, we investigated the impact of BCRP activity on the pharmacokinetics of drug transport across the placenta using dually perfused rat placenta. Transport of a model substrate of BCRP

- cimetidine - was studied in both materno-fetal and feto-maternal directions. To verify the specifity of BCRP-mediated transport two BCRP inhibitors GF120918 and fumitremorgin C were used. Our results demonstrated that BCRP plays two distinct roles in the kinetics of placental transport of drugs: 1) reduces the passage of drugs from the mother to the fetus and 2) actively accelerates the excretion of the drug already present in the fetal blood.

In the following two studies we investigated the expression and activity of the enzyme 11β-hydroxysteroid dehydrogenase (11β-HSD) which acts as a placental barrier to endogenous glucocorticoids (GC). In the first work we explored the role of placental 11β-HSD in the metabolism of GC in the course of rat pregnancy and the relationship between placental and fetal 11β-HSD activities and their effect on the ratio of active and inactive forms of GC in the fetal circulation. At first the expression and activity profiles of placental 11β-HSD type 1 and 2 during 13. and 21. day of pregnancy were studied. Expression of both types of 11β-HSD decreased at the end of pregnancy, however with different profiles. Dramatic decrease of mRNA levels of 11β-HSD1 was observed between 13. and 14. day of pregnancy followed by smaller reduction towards term. On the contrary, 11β-HSD2 was more abundantly expressed and decreased slowly from 13. to 21. day of gravidity. Decay of placental 11β-HSD2 in the last third of pregnancy was further confirmed on protein level. The decrease in 11β-HSD1 expression was followed by the drop in its activity. In contrast, an increase in NAD^+-dependent dehydrogenase activity of 11β-HSD2 was found in placental homogenates. These observations suggest the existence of an unknown posttranslational regulation mechanism which affects the activity of placental 11β-HSD2. The results from the functional studies with dithiotreitol *in-situ* revealed that this mechanism is presumably distinct from the process of activation and deactivation of 11β-HSD2 by reversible dimerization. Furthermore, the levels of corticosterone and 11-dehydrocorticosterone in the fetus were investigated and correlated with the activity of placental and fetal 11β-HSD. The results suggest that the activity of fetal 11β-HSD participates considerably on the regulation of GC levels in fetal blood, particularly at the end of pregnancy.

In the following study we examined the impact of antenatal GC administration on expression and conversion capacity of placental 11β-HSD type 2. Again rat placenta was chosen as an experimental model. Synthetic GC (dexamethasone and betamethasone) were administered to pregnant rats in two doses

(low or high) during 16. and 21. days of pregnancy. The expression of 11β-HSD in term placentas was analyzed by real-time RT-PCR and Western blotting. Conversion capacity was assayed by dually perfused rat placenta *in-situ* with corticosterone as a model substrate of 11β-HSD2. Our results showed that although the impact of antenatal steroid therapy on expression of 11β-HSD2 is negligible or, in the case of dexamethasone, limited to transcriptional level, conversion capacity of this enzyme is considerably decreased. The alteration in placental GC barrier was apparent not only at high doses of GC but also at low doses of dexamethasone. These observations suggest that synthetic steroids modulate the activity of 11β-HSD2 on the post-translational level. Furthermore, these results highlight the importance of functional analysis in the investigation of the effects which various exogenous compounds could have on the activity of placental enzymes.

VII.
SEZNAM PUBLIKOVANÝCH PRACÍ

1. Původní práce publikované v odborných časopisech

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