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Disertační práce

Endotelinový a renin – angiotenzinový systém, a jeho vztah k hypertenzi a hypertenznímu orgánovému postižení

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Obsah

Seznam zkratek použitých v textu

2K1C	Goldblattův model hypertenze (2K1C – 2 ledviny, 1 klipovaná)
ANG I	angiotenzin I
ANG II	angiotenzin II
big ET	big endotelin – prekurzor endotelinu.
DOCA – salt	Deoxycorticosteron acetát + sůl (model experimentální hypertenze)
ECE	endotelin konvertující enzym
ECE	endotelin konvertující enzym
ET	endotelinový systém
ET-1	endotelin 1
ET-2	endotelin 2
ET-3	endotelin 3
ET_A	endotelinový receptor typu A
ET_B	endotelinový receptor typu B
ET_{B1}	endotelinový receptor typu B1
ET_{B2}	endotelinový receptor typu B2
NEP	neutrální endopeptidáza
pre-pro-ET	preproendotelin; prekurzor endotelinu
Pro ET-1	proendotelin 1; prekurzor endotelinu
RAS	renin-angiotenzin-aldosteronovým systém
SHR	spontaneously hypertensiv rat; spontánně hypertenzních potkanů
SHR-SP	spontaneously hypertensiv rat – stroke prone; spontánně hypertenzní potkan
TGF β	transforming growth factor beta
TGR	transgenic rat; transgenní potkan
TGR(mRen2)27	transgenní kmen potkanů nesoucích myší gen pro renin
TNF α	tumor necrosis factor alfa

I. Úvod

Kardiovaskulární onemocnění jsou v České republice a v industrializovaných zemích častou příčinou morbidity a nejčastější příčinou mortality. V posledních několika desítkách let byl učiněn velký pokrok ve studiu kardiovaskulárních chorob a především byly učiněny zásadní změny v přístupu k léčbě těchto onemocnění. V poslední době je tento výzkum mimo jiné zaměřován také na studium cévní stěny. V roce 1988 popsal ve své práci Yanagisawa (Yanagisawa M. et al., 1988; Yanagisawa M. et al., 1998) nový peptid produkovaný endoteliálními buňkami, s velkým vazokonstrikčním potenciálem, a nazval jej endotelinem. Tento peptid o 21 aminokyselinách byl izolován z kultur aortálních endoteliálních buněk prasat. S postupujícím výzkumem se rozšiřovalo spektrum potenciálních účinků tohoto peptidu a jeho ubikvitérní zastoupení ve většině buněčných struktur organismu. Endotelinový systém dokáže v pikomolárních koncentracích potencovat účinek jiných vasokonstrikčních látek (Dohi Y. et al, 1992). Vzhledem k tomu se nabízí jeho použití jako možnost terapeutického zásahu zejména při léčbě arteriální hypertenze a hypertenzního orgánového postižení.

Endotelin působí spíše parakrinním a autokrinním způsobem než jako cirkulující hormon. Za zmínku stojí jeho blízká strukturální podobnost s jedem hadů čeledi zeměžmijovitých (Atractaspididae) produkujících ve svých jedových žlázách sarafotoxiny, které pro své silné vazokonstrikční a kardiotoxické účinky mohou způsobit i smrtelnou otravu člověka (Kloog Y. et al., 1989).

A. Tvorba a degradace endotelinových působků v organizmu

Biosyntéza endotelinových peptidů

V roce 1989 Inoue (Inoue A. et al., 1989) sekvenoval kompletní gen pro lidský endotelin. Necelý rok po té byly objeveny další dva strukturálně příbuzné peptidy. Endotelinový systém se tedy sestává z rodiny tří příbuzných peptidů Endotelinu 1 (ET-1), Endotelinu 2 (ET-2) a Endotelinu 3 (ET-3). Dodatečně byl ještě u myší a potkanů popsán peptid označovaný jako ET-4 nebo také „vasoactive intestinal constrictor". Ten je však identický s lidským endotelinem typu 2 (Bloch KD. et al., 1991).

Každý z izoenzymů endotelinu (ET 1-3) je produktem zvláštního genu, který kóduje jeho specifický prekurzor (Inoue A. et al, 1989). Endoteliny ET-2 a ET-3 se liší od původního peptidu ET-1 ve dvou, resp. v šesti aminokyselinách. Produktem přepisu ET genu je pre-pro-endotelin, který je tvořen přibližně 200 aminokyselinami (dle typu ET). Tento

endotelinový prekurzor je štěpen furinovou endopeptidázou a vzniká biologicky inaktivní peptid – „ big endotelin“ (big ET) o 37-41 aminokyselinách. Poslední fází je rozdělení big ET na pozici Trp_{21}-Val_{22} u ET1 a ET2 nebo na pozici Trp_{21}-Ile_{22} u ET3. Endoteliny působí v organismu přes své specifické receptory – ET_A a ET_B receptory.

Největší vazokonstrikční potenciál má ET-1, těsně následovaný ET-2 a nejméně účinný je v tomto směru ET-3. V experimentálním modelu na potkanech v anestézii dochází po bolusové intravenózní aplikaci ET-1 nebo ET-2 v dávce 1 nmol/kg k typické tlakové odpovědi. Tranzientní pokles arteriálního tlaku trvající 3-10 minut je následován postupným vzestupem tlaku nejméně na 1 hodinu. Nejprve převládne vazodilatační odpověď přes ET_B receptory, která vyvolá zvýšenou produkci oxidu dusnatého a prostacyklinu (PGI2) (Berti F. et al., 1993). Následná vazokonstrikční odpověď je způsobená stimulací ET_A receptorů hladkých svalových buněk cév. Tato typická tlaková odpověď nebyla pozorována u ET-3 (Bird JE. et al., 1993)

Endotelin konvertující enzym (ECE)

Za poslední krok při štěpení big ET je zodpovědný enzym ze skupiny metaloendopeptidáz. Byl nazván endotelin konvertujícím enzymem (ECE); sdílí funkční a strukturální podobnost s neutrální endopeptidázou a s proteiny krevních skupin Kell. Doposud byly popsány tři druhy ECE. Převažujícím místem vzniku Endotelinu 1 jsou endoteliální buňky cév. Na rozdíl od toho ET 2 a ET 3 byly nalezeny například ve dřeni ledvin a v nervových zakončeních. ECE může být částečně inhibován Phosphoramidonem.

Je známo několik subtypů ECE lišících se částečně svou aktivitou i zastoupením v různých orgánech.

Subtyp ECE-1 se nachází především na povrchu endotelových buněk cév, ale je prokazatelný také v buňkách hladkých svalů, exokrinních žláz nebo v nervových buňkách. Má vysokou aktivitu při neutrálním pH, je funkční jak intracelulárně tak na povrchu buněk. Afinita endotelin konvertujícího enzymu ECE-1 štěpit prekurzor endotelinu postupně klesá v tomto směru - big ET-1, big ET-2 a nejméně big ET-3. Mimo to dokáže ECE-1 štěpit i jiné biologické substráty, například bradykinin, substanci P, angiotenzin I, insulin a další (D´Orleans-Juste et al., 2003).

Existují čtyři izoformy ECE-1: ECE-1a, ECE-1b, ECE-1c a ECE-1d (Shimada K. et al., 1994; Schweizer A. et al., 1997; Valdenaire O. et al., 1999) Všechny isoformy jsou produktem přepisu stejného genu, lokalizovaného na chromozomu 1 (1p36), ale mají své specifické promotory. Tyto izoformy se liší svými N-terminálními konci a určuje se tak

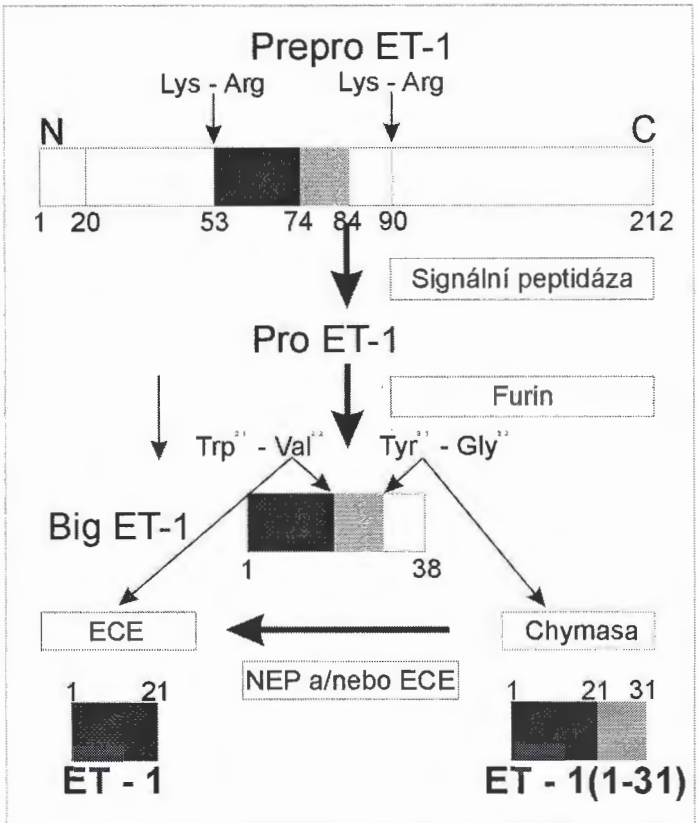
jejich lokalizace na buňce. ECE-1a, ECE-1c a ECE-1d jsou z plazmatické membrány orientovány svou katalytickou doménou vně buněk. Aktivita ECE-1b je orientována intracelulárně. V roce 2003 popsal Muller (Muller L. et al., 2003) aktivitu ECE-1b a ECE-1d endosomálně.

Je známo, že ECE-2 je z 59% identický s ECE-1 a má stejně tak 4 izoformy s podobnou lokalizací v buňce. Nejvyšší aktivitu vykazuje při pH 5.8, proto je aktivní spíše intracelulárně (Emoto N. et al., 1995). Můžeme ho nalézt například na neuronech nebo buňkách nadledvin.

Třetí ze zástupců rodiny endotelin konvertujících enzymů ECE-3 byl izolován z mikrosomů bovinních duhovek (Hasegawa H. et al., 1998). ECE-3 je specifický pouze pro big ET-3 a neštěpí big ET-1 ani big ET-2.

Obrázek A1: *Schéma syntézy ET-1$^{1-21}$ a ET-1$^{1-31}$*

Důležitost ECE lze dokumentovat asi 140-ti násobným zvýšením vazokonstrikční účinnosti ET1 oproti prekurzoru big ET. Pro-endotelin pak nevykazuje téměř žádnou vasomotorickou aktivitu (Rubanyi GM. et al., 1994) (Obrázek A1). Významná fyziologická úloha endotelin konvertujících enzymů je patrna i u modelu homozygotní (-/-) knock-out myši pro gen ECE-1, u které tato genová konstelace vede k fatálním vývojovým defektům



(Legenda: Prepro ET-1: Preproednotelin 1; Pro ET-1: Proendotelin; ECE: Endotelin konvertující enzym; NEP: neutrální endopeptidáza; upraveno dle: D´Orleans-Juste et al., 2003)

Alternativní cesta syntézy endotelinu

Jako alternativní cesta štěpení Angiotenzinu I (ANG I) na Angiotenzin II (ANG II) působí v lidském myokardu chymása (chymosin) žírných buněk. Stejně tak byl popsán i alternativní způsob syntézy endotelinu pomocí tohoto enzymu. Tímto mechanismem je štěpen big ET1 a big ET2 v místě vazby Tyr_{31}-Gly_{32}, čímž vzniká nový peptid s vazokonstrikční aktivitou ET-1 (1-31), respektive ET-2(1-31). V dalším kroku je ET-1 (1-31) rozštěpen především pomocí neutrální endopeptidázy (NEP), ale také ECE, na finální ET-1 (1-21). In vitro způsobuje podání Endotelin-1 (1-31) pomalejší, ale déle trvající vazokonstrikční odpověď, bez typického bifázického průběhu (Fecteau MH et al., 2005).

Protože u celé řady srdečních, plicních a cévních chorob dochází k degranulaci žírných buněk, dá se předpokládat účast ET-1 (1-31) v rozvoji těchto onemocnění (Doggrell SA. et al., 2004).

Faktory regulující syntézu endotelinu

Regulace tvorby endotelinu se odehrává hlavně na úrovni syntézy, především ve fázi transkripce. Zvýšená hladina mRNA ET-1 byla pozorována jako odpověď na podání ANGII, noradrenalinu, vazopresinu nebo trombinu. Také cytokiny (tumor necrosis factor alfa (TNF α), interleukiny a transforming growth factor beta (TGF β)) zvyšují syntézu ET-1 (Yanagisawa M. et al., 1989; Kedzierski RM. et al., 2001; Hynynen MM. et al., 2006). Hladiny mRNA ET-1 jsou zvyšovány hypokapnií a snižovány hypoxií (Yoshimoto S. et al., 1991).

V epiteliálních buňkách smykové napětí nejprve zvyšuje a následně snižuje produkci endoteliální mRNA (Malek AM. et al., 1999). Snížené hladiny mRNA ET-1 byly pozorovány jako odpověď na NO, prostacyclin nebo atriální natriuretický faktor (ANF) (Kohno M. et al., 1992; Kedzierski RM. et al., 2001; Hynynen MM. et al., 2006). V pozitivním smyslu se uplatňují i adhezivní molekuly ICAM-1, VCAM-1.

Endoteliální buňky obsahují podlouhlé vezikuly nazvané Weibel-Paladeho tělíska, která slouží jako zásobárna pro ET-1. Při aktivaci endoteliálních buněk dochází k přesunu těchto organel k povrchu buňky, následně dojde k fúzi s plazmatickou membránou a exocytóze obsahu tělísek vně buňky (van Mourik JA et al., 2002).

Převažujícím místem vzniku ET-1 jsou endoteliální buňky cév, ale může být produkován leukocyty, makrofágy, hladkými svalovými buňkami, kardiomyocyty nebo mesangiálními

buňkami. Na rozdíl od ET-1 byly ET-2 a ET-3 nalezeny ve dřeni ledvin, nadledvinách nebo v nervových zakončeních.

Katabolismus endotelinů

Jednu z nejdůležitějších cest odbourávání endotelinů zajišťují ET_B receptory. Po navázání Endotelinu na tento receptor dochází k internalizaci tohoto komplexu a jeho degradaci. Děje se tak především v plicích a dále pak v ledvinách. Asi 80% ET-1 je odstraňováno z plazmy právě ET_B zprostředkovanou cestou v plicních cévách (Fukuruoda T. et al., 1994).

Clearance endotelinů je složitý proces, při kterém se dále jako další cesta degradace uplatňuje již zmíněný hydrolytický enzym - NEP (Abassi ZA. et al., 1992). Aktivita tohoto enzymu byla pozorována v plicích, v ledvinách, ale také u buněk karcinomu endometria (Suzuki T. et al. 2001). V tomto případě aktivita NEP a tím i ET-1 koreluje se stupněm nádorového postižení (tumor grade).

B. Mechanismus účinků

Do současnosti byly popsány dva druhy endotelinových receptorů, označených ET_A a ET_B (Sakurai T. et al., 1990). Receptory endotelinů působí přes své G-proteiny, aktivují tak fosfolipázu C, zvyšují intracelulární kalcium a indukují časné geny. Receptor ET_A působí vazokonstrikčně, zvyšuje buněčnou proliferaci a má pozitivně inotropní efekt. Má subnanomolární afinitu pro ET-1 a ET-2 a o dva řády nižší aktivitu pro ET-3. Receptor ET_A se vyskytuje na endoteliích cév, na epiteliích průdušek, kardiomyocytech, hepatocytech, neuronech a dalších buněčných strukturách.

Receptor ET_B se vyskytuje na cévních endoteliích, hladkých svalových buňkách cév, makrofázích, hepatocytech, tubulárních buňkách ledvin, neuronech. Hlavní funkcí ET_B receptorů je uvolnění NO, prostacyklinu a ovlivnění buněčné apoptózy (Rich S. and McLaughlin V.V. et al., 2003). Lokalizace receptorů pro endotelinový systém se na celé řadě buněčných struktur tedy překrývá a některé buňky mají oba typy receptorů. Výsledný efekt endotelinů je tedy závislý na potenciálním zastoupení jednotlivých receptorů v daném vaskulárním řečišti.

Fyziologická úloha endotelinů v ontogenezi

Při defektu genů pro ET-1, ET_A a ECE-1 dochází v experimentu k vývojovým anomáliím hlavových a srdečních výtokových struktur (pravostranný srdeční oblouk, komorové

septální defekty). Vývojové vady u jedinců těchto myších linií jsou neslučitelné se životem a zvířata umírají těsně po porodu pro asfyxii z důvodu závažných malformací obličejových a krčních struktur (rozštěpové vady patra, hypoplastická mandibula nebo hypoplastický jazyk).

Experimenty s genově modifikovanými zvířecími modely přinesly celou řadu důkazů o důležitosti endotelinového systému v ontogenezi savců. Myší modely s knock-out geny pro ET-3, ET_B a ECE ukázaly nezbytnost těchto genových produktů pro správný embryonální vývoj struktur odvozených z neurální trubice.

Defekt genu pro ECE-1 vede u těchto zvířat k deficitu ET-1/ET_A a současně ET-3/ET_B, což vyústí v nedostatečnou tvorbu melanocytů (normální výskyt retinálního pigmentu, ale bílé zbarvení srsti „white spotting"). Zároveň dochází k chybnému vycestování nervových buněk do nervových ganglií - v gastrointestinálním traktu je pak nedostatečná střevní inervace. Vyvíjí se dilatace proximálního tračníku a zvířata pro střevní obstrukci postupně umírají (analogie Hirschsprungovy choroby). Detailněji je tato problematika popsána v přehledném článku autorů Kadziersky et al., 2001.

Endoteliny v cévách různých orgánových systémů

Z rodiny endotelinových peptidů má z hlediska hypertenze a hypertenzního orgánového poškození nejdůležitější úlohu ET-1. Postupně byly vyvíjeny antagonisté endotelinových receptorů se schopností neselektivně či selektivně blokovat ET receptory. Přehled antagonistů endotelinových receptorů je uveden v Tabulce A1.

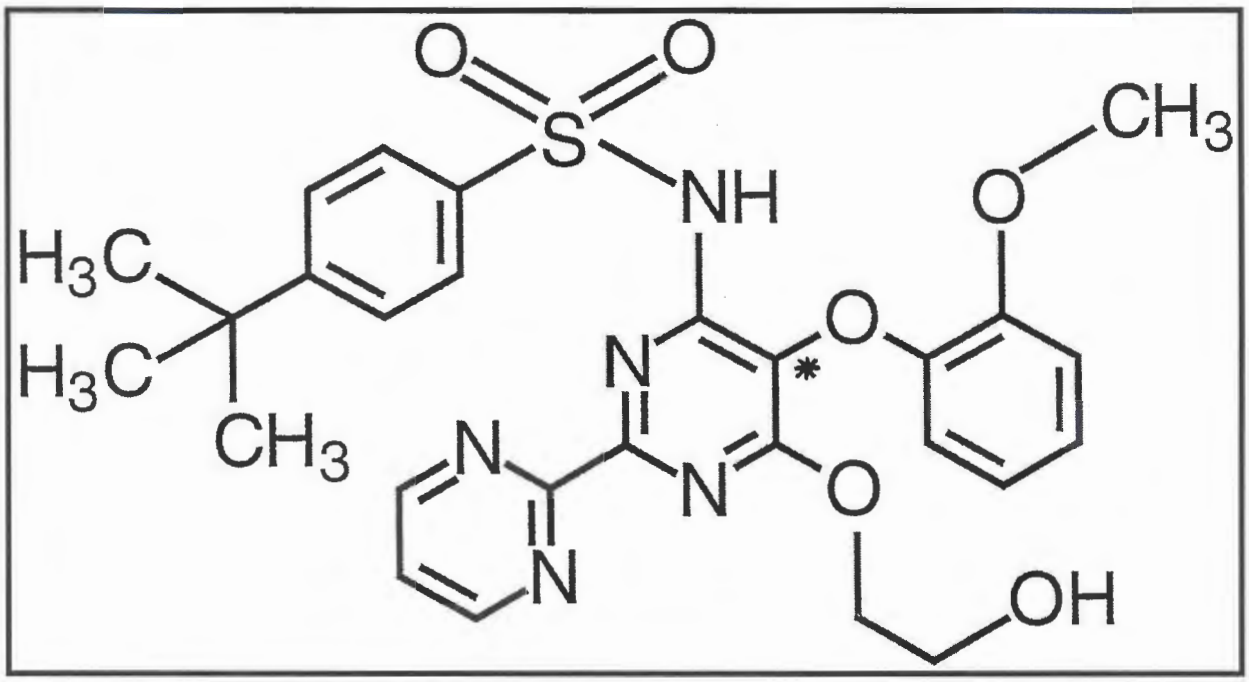
Tabulka A1: *Přehled selektivních a neselektivních endotelinových blokátorů*

ET_A / ET_B	ET_A	ET_B
TAK-044	BQ-123	BQ-788
Bosentan (Tracleer®)	BQ-610	REA-701-1
PD 145065	FR139317	RO-468443
L-744, 453	IPI-725	
L-751281	A-127722.5	
L-754, 142	LU135252	
SB209670	PD155080	
SB217242	PD156707	
	BMS-182874	
	TBC11251	
	Atrasentan	

(Upraveno dle: Kedzierski et al., 2001)

Tento fakt výrazným způsobem pomohl ve studiu endotelinového systému jak na zvířecích modelech, tak později i v klinické praxi. V současné době jsou neselektivní blokátory ET_A a ET_B (Bosentan chemický vzorec a struktura viz. obrázek A2) a selektivní blokátory ETA již ve fázi II klinického zkoušení v léčbě plicní hypertenze a chronického srdečního selhání. Dosud se nepodařilo najít klinické použití pro selektivní blokátor receptoru ET_B.

Obrázek A2: *Chemická struktura neselektivního endotelinového blokátoru – Bosentan (4-tert.-butyl-N-[6-(2-hydroxyethoxy)-5-(2-methoxyphenoxy)-2,2'-bipyrimidine-4-yl]benzenesulfonamide)*



(Upraveno dle publikace Weber C. et al., 1999)

Bylo zjištěno, že většina pacientů s arteriální hypertenzí má normální hladiny plazmatického Endotelinu-1. Výjimku tvoří pacienti s renálním selháním nebo vzácné případy hemangioendotheliomu, u kterých je hladina ET-1 v plazmě několikanásobně zvýšená. Vysvětlením těchto nálezů může být již zmiňovaný fakt, že ET-1 působí především parakrinním nebo autokrinním mechanismem. Koncentrace ET-1 v cévní stěně je asi stonásobně vyšší než je jeho koncentrace v plazmě.

V úvodu bylo již zmíněno, že intravenózní aplikace bolusu ET-1 vede ke krátkodobému poklesu krevního tlaku, po kterém následuje déletrvající vzestup tlaku. Počáteční pokles cévní rezistence je dán účinkem NO a prostacyklinu, ke kterému dojde stimulací ET_B receptorů endoteliálních buněk. Druhá, vazokonstrikční fáze je pak způsobena stimulací ET_A receptorů na hladkých svalových buňkách a zvýšením jejich tonu.

V geneticky změněném myším modelu s nedostatkem ET-1 (ET-1 knock-out gene) byly naměřeny o 15 torrů nižší systolické i diastolické tlaky než u kontrolní skupiny zvířat. Vliv dalších faktorů ovlivňujících krevní tlak u tohoto zvířecího modelu byl odstraněn farmakologickou blokádou angiotenzinového a sympatického systému.

Endotelinový systém má důležitou úlohu i v patofyziologii aterosklerózy. U králíků krmených vysokocholesterolovou dietou dochází k 2-4 násobnému vzestupu tkáňových hladin ECE-1 a až k šestinásobnému zvýšení tkáňových hladin ET-1 (Mitani H. et al., 2000).

Pomocí radioizotopově značeného ET-1 podávaného intravenózně byl podán důkaz o přítomnosti endotelinových receptorů v aterosklerotických plátech (Dashwood MR. et al., 1993).

Endotelin-1 je také hlavním srdečním endotelinem, jehož zdrojem jsou především kardiomyocyty, endoteliální buňky a srdeční fibroblasty. In-vitro byl pozorován pozitivně inotropní a chronotropní efekt ET-1. Během několika hodin po závažné koronární ischémii jsou několikanásobně zvýšené hladiny plazmatického ET-1. Účinky endotelinové systému a blokátorů ET receptorů byly studovány na zvířecích modelech akutního infarktu myokardu (IM). Ze závěrů těchto studií vyplývá, že aktivace ET receptorů je výhodná několik hodin po IM a naopak z dlouhodobého hlediska se pravděpodobně aktivovaný endotelinový systém podílí na rozvoji městnavého srdečního selhání.

V plicích je ET-1 exprimován na endoteliích cév, epiteliích bronchiálního stromu a na makrofázích. Endotelinový systém se podílí na regulaci tonu dýchacích cest a je proto pravděpodobná jeho spoluúčast při vzniku intersticiálních plicních procesů a astmatu. U zvířecího modelu krys exprimujících ve zvýšené míře ET-1 dochází k vývoji septovaných edematózních alveolů a k hyperplázii pojivové tkáně. Dle histologického vyšetření onemocnění připomíná obliterující bronchiolitidu.

Velice důležitá je fyziologie a patofyziologie působení ET-1 v plicních cévách. Vazokonstrikční působení na plicní cévy je zprostředkováno ET_A receptory. U pacientů s primární plicní hypertenzí jsou v endotéliích plicních artérií zvýšené koncentrace ET-1 mRNA a ET-1 (Giaid A. et al., 1993). Zůstává otázkou, do jaké míry se na zvýšených hladinách ET-1 podílí jeho zvýšená produkce (mRNA) nebo naopak snížená degradace pomocí ET_B receptorů. V oblasti plicní hypertenze bylo zatím dosaženo největšího klinického použití endotelinových blokátorů (viz níže).

Taktéž v ledvinném parenchymu se uplatňuje endotelinový systém. ET-1 je syntetizován jako u jiných orgánových systémů endoteliálními buňkami cév, ale zároveň jsou ET-1 a ET-3 produkovány epiteliálními buňkami dřeňových a korových sběrných kanálků. V ledvinném parenchymu se vyskytují oba typy endotelinových receptorů, avšak v tubulech ledvin převažuje ET_B typ receptoru s maximálním výskytem v oblasti sběrných kanálků vnitřní dřeně. Endotelinový systém výrazným způsobem ovlivňuje průtok krve ledvinou, reabsorpci vody a acidobazickou rovnováhu.

Endotelin-1 působením přes ET_A receptory snižuje průtok krve ledvinami, kortikální perfúzi, glomerulární filtraci a sodíkovou exkreční frakci. Naopak stimulace ET_B receptorů ET-1 ve dřeni zvyšuje diurézu a natriurézu.

Vzhledem k úzké souvislosti endotelinového sytému s endoteliemi cév je jasné, že bude tento systém ovlivňovat většinu fyziologických a patofyziologických procesů ve všech orgánech. Podrobné informace o těchto systémech jsou uvedeny v souborném článku Kadzierski et al., 2001.

C. Interakce mezi endotelinovým a renin – angiotenzinovým systémem

Endotelin a endotelinové blokátory v experimentálních modelech hypertenze

Vztahy mezi renin-angiotenzin-aldosteronovým (RAS) a endotelinovým (ET) systémem představují důležitý prvek v rozvoji arteriální hypertenze a s tím spojeného hypertenzního orgánového. Potenciální mechanismy, kterými ET-1 ovlivňuje produkci reninu, jsou znázorněny na obrázku A3 (Rossi GP. Et al., 1999).

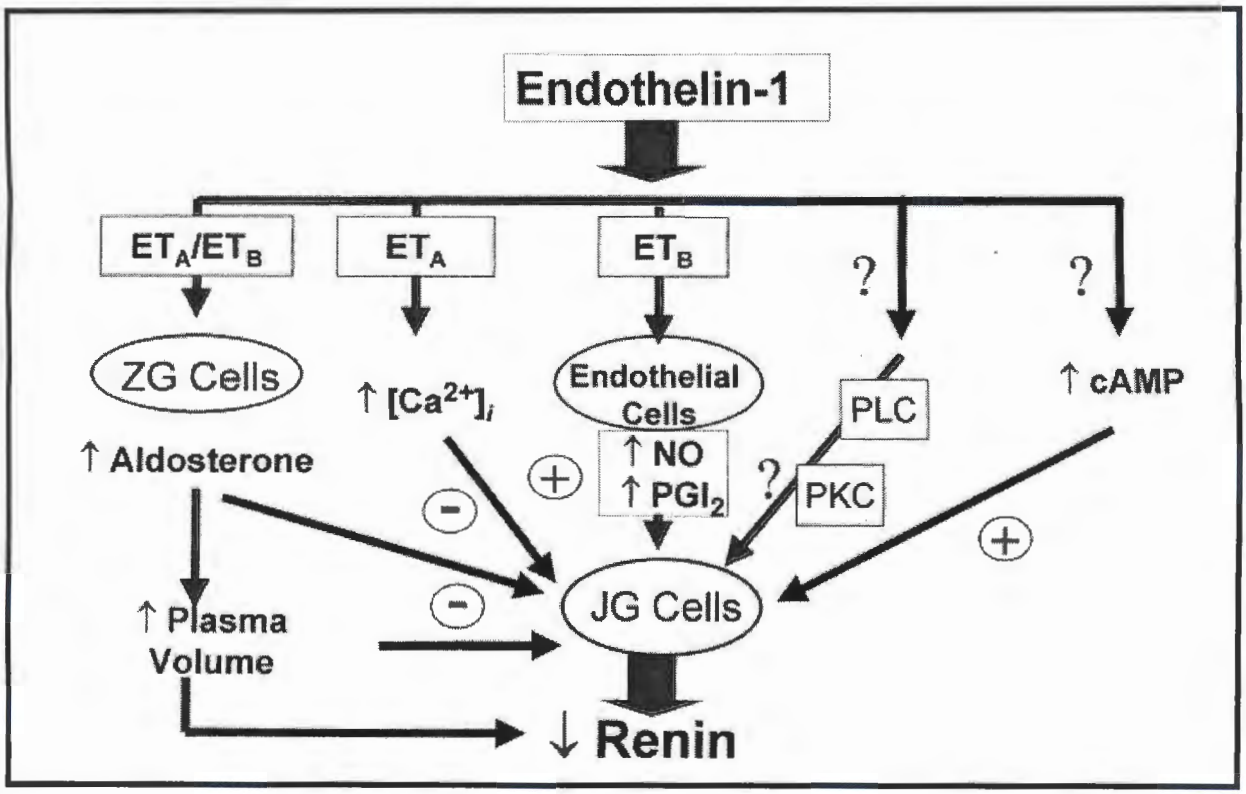
První studie v experimentálním modelu hypertenze provedené u kmenů spontánně hypertenzních potkanů (SHR) a u spontánně hypertenzních potkanů se sklony k iktu (SHR-SP) ukázaly, že po akutním podání ET_A blokátoru u SHR-SP potkanů dochází na rozdíl od SHR potkanů k poklesu krevního tlaku. Taktéž neselektivní blokáda ET_A/ET_B receptorů Bosentanem u SHR potkanů neovlivňuje vývoj hypertenze ani hypertenzního orgánového postižení (Jin-S Li et al., 1995; Moreau P. et al., 2003; Cosenzi A. et al., 1999).

Naopak chronická blokáda ET_A a ET_B receptorů pomocí Bosentanu u jiného modelu hypertenze – u DOCA-salt potkanů (Deoxycorticosteron acetát + sůl) snižuje krevní tlak.

Důležitost renin-angiotensinového systému pro rozvoj hypertenze a hypertenzního orgánového poškození u různých modelů experimentální hypertenze byla již dobře prostudována (Bader M. et al., 2001). Předcházející studie provedené postupně několika

autory prokázaly, že endotelinový systém hraje důležitou úlohu v patogenezi u sůl senzitivních modelů hypertenze a jejich hypertenzního orgánového postižení (Moreau P. et al, 2003; Roux S. et al., 1999). Blokáda endotelinových receptorů se ukázala být účinnou především u „sůl senzitivních" modelů hypertenze – již zmiňovaný DOCA-salt, dále Dahl - sůl senzitivní potkan, SHR-SP se zvýšeným přísunem soli v potravě. Z tohoto důvodů vzbuzuje interakce mezi RAS a ET systémem zvýšenou pozornost. Bylo prokázáno, že ANG II stimuluje pre-pro-ET mRNA expresi a ET-1 uvolnění v in-vitro kulturách endoteliálních buněk (Emori T. et al., 1989), v buňkách hladkých svalů (Sung CP. et al., 1994) a v mesangiálních buňkách ledvin (Kohno M. et al., 1992). Navíc bylo prokázáno, že u externě dodávaného ANG II (ANG II-infused hypertensive rats) stoupá ledvinná exprese pre-pro-ET mRNA a zvyšuje se koncentrace ET-1 (Alexander BT. et al., 2001; Sasser JM. et al., 2002).

Obrázek A3: *Mechanismy ovlivňující produkci reninu endotelinem 1.*



(ZG – buňky zona glomerulosa; JG – juxtaglomerulární buňky; PLC – fosfolipáza C; PKC – proteinkináza C; PGI_2 – prostaglandin I; cAMP – cyklický adenosin monofosfát; upraveno dle: Rossi GP. et al., 1999)

Avšak u ANG II dependentního modelu hypertenze in-vivo tyto studie nepřinesly jednoznačné výsledky. Bylo zjištěno, že blokáda ET receptorů u ANG II infundovaných potkanů jednoznačně zpomaluje rozvoj hypertenze a snižuje poškození ledvin a srdce (Ficai S. et al., 2001; d´Uscio LV. et al., 1997). Také podávání Bosentanu (neselektivního

blokátoru receptorů AT_A/ET_B) dvojitě transgenním potkanům nesoucím geny pro lidský renin a angiotenzin s rozvojem maligní hypertenze snížilo krevní tlak, redukovalo mortalitu, albuminurii, poškození ledvin a srdeční hypertrofii (Muller DN. et al., 2000). V některých studiích se naopak nepodařilo prokázat, že blokáda ET receptorů pomáhá předcházet rozvoji hypertenze a přidružených kardiovaskulárních komplikací. Například u Goldblattova modelu hypertenze (2K1C – dvě ledviny, jedna klipovaná) blokáda ET_A receptoru nesnižuje krevní tlak (Ehmke H. et al., 1999; Saam T. et al., 2003;.Touyz RM. et al., 2000; Blezer ELA. et al., 1999).

Pokud tedy shrneme tyto výsledky, tak u hypertenzních zvířecích modelů s exogenně dodávaným ANG II lze pozorovat endotelin dependentní složku hypertenze, zatímco u modelů s endogenní produkcí ANG II tato složka pozorována nebyla. Bohužel důvod tohoto rozporu byl dosud nejasný.

Z tohoto důvodu jsme se v našich studiích rozhodli použít ANG II dependentní model hypertenze – transgenní kmen potkanů nesoucích myší gen pro renin (TGR; označení kmene - TGR(mRen2)27). Rozvoj hypertenze u tohoto kmene potkanů je tedy dán inzercí myšího genu pro renin do genomu potkana (Mullins J. et al., 1990). Tito TGR potkani tedy reprezentují model hypertenze s dobře definovaným genetickým pozadím, u kterých je rozvoj hypertenze dán alterací jednoho genu a je jasně ANG II závislý (Langheinrich M. et al., 1996). Přehled vlivu endotelinového systému u různých experimentálních modelů hypertenze je znázorněno v tabulce A2.

Na základě uvedených studií se dá tedy předpokládat, že vzájemné působení ET-1 a ANG II bude mít důležitou úlohu v rozvoji maligní fáze hypertenze (Mullins J. et al., 1990; Langheinrich M. et al., 1996).

Předmětem studia těchto interakcí byl i výzkum prováděný naší pracovní skupinou. Stanovili jsme si za cíl prokázat vliv neselektivní blokády ET_A a ET_B receptorů u jasně definovaného modelu hypertenze, tedy homozygotních samčích TGR(mRen2)27 potkanů, na vliv rozvoje hypertenze a hypertenzního orgánového poškození (Dvorak P. et al., 2004) V další studii na stejném modelu arteriální hypertenze jsme se zaměřili na vliv vysokoslané diety na tyto transgenní zvířata (Opocensky M. et al., 2004).

V recentně publikované studii byla zaměřena pozornost na efekt časné blokády ET_A receptorů pomocí Atrasentanu (Atrasentan - ABT-627) na rozvoj hypertenze, hypertenzního orgánového postižení a mortality (Vaneckova I. et al., 2005). Výsledky těchto studií jsou součástí této disertační práce.

Tabulka A2: *Efekt blokády endotelinového systému na kontrolu arteriální hypertenze u jednotlivých experimentálních modelů vysokého krevního tlaku.*

Efektivita antihypertenzní léčby endotelinovými blokátory v experimentálních modelech hypertenze	
Snížení TK	Publikace
+++	
DOCA - sůl	Karam et al. 1996; Li et al 1994; Matsumura et al. 1999; Polock et al. 2000
Aldosterone + sůl	Park a Schiffrin 2001
Dahl SS	Barton et al. 1998; d´Uscio et al. 1997; Kassab et al. 1998; Okada et al. 2000
SHR-SP	Blezer et al. 199; Chillon et al. 1996; Sharifi et al. 1998; Stasch et al. 1995; Touyz et al. 2000
Angiotensin II	Herizi et al. 1998; Moreau et al. 1997
+	
2K-1C	Bianciotti and Bold 2001; Ehmke et al. 1999; Hocher et al. 1999; Li et al. 1996

SHR	Karam et al. 1996; Li a Schiffrin 1995
L-Name	Fortepiani et al. 1999; Moreau et al. 1997; Sventek et al. 1997; Tharaux et al.1999
1K-1C	Li at al. 1996
mREN2	Rossi et al.; Dvorak et al. 2004, Opocenský et al 2005, Vaneckova et al. 2005
hREN-hAGT	Bohlender et al. 2000

(Upraveno dle: Moreau P. et al., 2003)

II. Experimentální studie naší pracovní skupiny na modelu hypertenze potkanů s vloženým myším genem pro renin Ren-2 renin gene (TGR; jméno kmene).

II A. Blokáda endotelinových receptorů zmírňuje hypertenzní orgánové postižení u Ren-2 transgenních potkanů

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Blockade of Endothelin Receptors Attenuates End-Organ Damage in Homozygous Hypertensive Ren-2 Transgenic Rats

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Key Words
Hypertension · Endothelin · Renin-angiotensin system · Bosentan · End-organ damage

Abstract
Background/Aims: A growing body of evidence suggests that the interplay between the endothelin (ET) and the renin-angiotensin systems (RAS) plays an important role in the development of the malignant phase of hypertension. The present study was performed to evaluate the role of an interaction between ET and RAS in the development of hypertension and hypertension-associated end-organ damage in homozygous male transgenic rats harboring the mouse Ren-2 renin gene (TGRs) under conditions of normal-salt (NS, 0.45% NaCl) and high-salt (HS, 2% NaCl) intake. ***Methods:*** Twenty-eight-day-old homozygous male TGRs and age-matched transgene-negative male normotensive Hannover Sprague-Dawley (HanSD) rats were randomly assigned to groups with NS or HS intake. Nonselective $ET_{A/B}$ receptor blockade was achieved with bosentan (100 mg/kg/day). Systolic blood pressure (BP) was measured in conscious animals by tail plethysmography. Rats were placed into metabolic cages to determine proteinuria and clearance of endogenous creatinine. At the end of the experiment the final arterial BP was measured directly in anesthetized rats. Kidneys were taken for morphological examination. ***Results:*** All male HanSD fed either the NS or HS diet exhibited a 100% survival rate until 180 days of age (end of experiment). The survival rate in untreated homozygous male TGRs fed the NS diet was 41%, which was markedly improved by treatment with bosentan to 88%. The HS diet reduced the survival rate in homozygous male TGRs to 10%. The survival rate in homozygous male TGRs on the HS diet was significantly improved by bosentan to 69%. Treatment with bosentan did not influence either the course of hypertension or the final levels of BP in any of the experimental groups of HanSD rats or TGRs. Although the ET-1 content in the renal cortex did not dif-

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fer between HanSD rats and TGRs, ET-1 in the left heart ventricle of TGRs fed the HS diet was significantly higher compared with all other groups. Administration of bosentan to homozygous male TGRs fed either the NS or HS diet markedly reduced proteinuria, glomerulosclerosis and attenuated the development of cardiac hypertrophy compared with untreated TGR. ***Conclusions:*** Our data show that nonselective $ET_{A/B}$ receptor blockade markedly improves the survival rate and ameliorates end-organ damage in homozygous male TGRs without significantly lowering BP.

Introduction

The renin-angiotensin (RAS) and endothelin (ET) systems represent the two most powerful vasoconstrictor systems known to date [for review, see 1, 2]. The important role of the RAS in the pathophysiology of hypertension and in the development of hypertensive end-organ damage is well recognized [3]. Previous studies have demonstrated that the ET system also plays an important role in the pathogenesis of salt-sensitive models of hypertension and in associated end-organ damage [for review, see 2, 4]. Therefore, the interaction between the RAS and ET system has received increasing attention. It has been reported that angiotensin II (ANG II) stimulates preproET mRNA expression and ET-1 release in cultured endothelial [5], smooth muscle [6] and renal mesangial cells [7]. In addition, it has been shown that renal expression of preproET mRNA and the ET-1 concentration are enhanced in ANG II-infused hypertensive rats [8, 9].

However, in vivo studies in ANG II-dependent models of hypertension did not yield consistent results. On one hand, it has been shown that ET receptor blockade markedly attenuated the development of hypertension and ameliorated renal and cardiac damage in ANG II-infused hypertensive rats [10, 11; for review, see 2]. Furthermore, administration of bosentan, a nonselective blocker of ET_A and ET_B receptors, lowered blood pressure (BP) and reduced the mortality rate, albuminuria, renal injury and cardiac hypertrophy in double transgenic malignant hypertensive rats harboring both human renin and angiotensinogen genes (dTGR) [12]. On the other hand, there are studies that failed to demonstrate that ET receptor blockade prevents the development of hypertension and related cardiovascular damage [for review, see 2]. Thus, it has been shown that in two-kidney, one-clip (2K1C) Goldblatt hypertensive rats ET_A receptor blockade does not lower BP [13, 14]. Taken together, these studies suggest that hypertensive models induced by exogenously administered ANG II exhibit an ET-dependent component, while models with enhanced endogenous production of ANG II do not [for review, see 2]. However, the reason(s) for this discrepancy in response to ET blockade remain(s) unclear at present. In order to determine the contribution of an interaction between ET-1 and ANG II to the development of hypertension and related end-organ damage in an ANG II-dependent model of hypertension, we utilized a rat strain transgenic for the mouse Ren-2 renin gene (TGR; strain name TGR(mRen2)27). The development of hypertension in this strain is a result of insertion of the mouse Ren-2 renin gene into the rat genome [15]. Thus, TGRs represent a model of hypertension with a well-defined genetic background in which the development of hypertension can be attributed to a single gene alteration and is clearly ANG II-dependent [for review, see 16].

In view of the growing body of information suggesting that the interplay between ET-1 and ANG II plays an important role in the malignant phase of hypertension [17, 18; for review, see 2], the first aim of the present study was to evaluate the effects of bosentan treatment on the course of hypertension in homozygous male TGRs which are generally considered as a model of malignant hypertension [for review, see 15, 16].

Since it has been reported that TGRs also show a salt-sensitive component of hypertension [19], the second aim of this study was to delineate whether high-salt intake would accelerate the course of hypertension and end-organ damage in this model.

Materials and Methods

The protocols in the present study were designed according to the Guiding Principles in the Care and Use Animals approved by the Council of the American Physiological Society and were approved by Czech Animal Care and Use Committee (protocol 79#2001).

Animals

Experiments were performed on homozygous male TGRs and age-matched transgene-negative male Hannover Sprague-Dawley rats (HanSDs). All animals used in the present study were bred at the Center for Experimental Medicine of the Institute for Clinical and Experimental Medicine from stock animals supplied from the Max Delbrück Center for Molecular Medicine of Berlin, Germany. Animals were kept on a 12/12-hour light/dark cycle.

Diets

All diets used in the present study were produced by SEMED (Prague, Czech Republic). Rats were fed either a normal-salt diet (NS; 0.45% NaCl, 19–21% protein) or a high-salt diet (HS; 2% NaCl,

Table 1. Experimental groups of rats

Group	n	Group description
HanSD + NS	13	male HanSD fed a normal-salt diet
HanSD + NS + bosentan	14	male HanSD fed a normal-salt diet and treated with bosentan
HanSD + HS	14	male HanSD fed a high-salt diet
HanSD + HS + bosentan	14	male HanSD fed a high-salt diet and treated with bosentan
TGR + NS	34	homozygous male TGR fed a normal-salt diet
TGR + NS + bosentan	32	homozygous male TGR fed a normal-salt diet and treated with bosentan
TGR + HS	31	homozygous male TGR fed a high-salt diet
TGR + HS + bosentan	32	homozygous male TGR fed a high-salt diet and treated with bosentan

HanSD = Transgene-negative rats; TGR = homozygous transgenic rats, (mRen2)27; NS = normal-salt diet; HS = high-salt diet.

19–21% protein). Bosentan (Actelion, Allschwil, Switzerland) was mixed to the NS and HS diets at a concentration depending on the food intake so that the final consumption of bosentan was 100 mg/kg body weight (BW)/day. This dose of bosentan was chosen based on previous studies that demonstrated a maximal pharmacological effect in ANG II-infused hypertensive rats and in the models of salt-sensitive hypertension [10, 12; for review, see 2, 4]. In preliminary experiments, such a dose of bosentan completely blocked BP responses to an intravenous (i.v.) bolus dose of ET-1 (250 ng) in homozygous male TGRs as well as HanSDs treated with bosentan for 2 days. In addition, separate groups of 10-week-old homozygous male TGRs (n = 7) and HanSDs (n = 6) were given bosentan at a dose of 200 mg/kg BW/day for 10 days to ensure that any increase in dose would not affect BP compared with animals fed the recommended dose of bosentan.

Experimental Design and Functional Examination

Twenty-eight-day-old homozygous male TGRs and age-matched male HanSDs from several litters were randomly assigned to experimental groups, taking care that animals from a single litter did not prevail in any of the groups. The experimental groups are shown in table 1.

Systolic blood pressure (SBP) was measured in conscious animals by tail plethysmography from 29 to 40 days of age every 2 days, from 41 to 60 days of age every 3 days, and thereafter every week until the end of the experiment (180 days of age). On each occasion BP was determined as the mean of 4 measurements. This method was previously validated in our laboratory by Heller et al. [20]. At 40, 80, 120 and 170 days of age, rats were placed individually into metabolic cages (after at least 4 days of training to accustomize them) and their 24-hour urine was collected for protein excretion and calculation of clearance of endogenous creatinine (a blood sample for determination of the plasma concentration of creatinine and electrolytes was taken in the morning of the 2nd day). BWs were obtained on the day of SBP measurements, and from day 60 until the end of the experiment rats were weighted additionally twice a week. Food intake was monitored once a week and thereafter the concentration of bosentan in the food was modified in order to reach a daily bosentan intake of 100 mg/kg BW. At the end of the experiments, animals were anesthetized with thiopental sodium (50 mg/kg BW), and the right carotid artery was cannulated with PE-50 tubing for direct measurement of mean arterial pressure (MAP).

Morphological Examination

The ratios of heart weight (HW, mg)/BW (g) and right kidney weight (KW, mg)/BW (g) were used as indices of cardiac hypertrophy (HW/BW) and kidney hypertrophy (KW/BW), respectively. The left kidney was quickly removed, fixed in 4% formaldehyde, dehydrated and embedded. Paraffin sections were stained with hematoxylin eosin and periodic acid-Schiff reaction (PAS). Slides were evaluated in a blind fashion. Fifty glomeruli in each kidney were examined on a semiquantitative scale as described previously [21]: grade 0 = all glomeruli normal; grade 1 = 1–2 glomeruli affected; grade 2 = more than 2 but less than 17 glomeruli affected; grade 3 = 17 or more glomeruli affected.

Tissue ET-1 Concentration

In separate groups of male HanSDs and homozygous male TGRs fed either the NS or HS diet (n = 9 in each group) from 28 to 56 days of age, the kidney cortex and left ventricular heart ET-1 concentrations were examined. The purpose of this protocol was to evaluate whether tissue ET-1 levels are increased before the transition to the malignant phase of hypertension in this model. Therefore, this protocol was chosen because at this time point (56 days of age) every group still exhibited a 100% survival rate. ET-1 concentrations were determined by ELISA (Amersham, Braunschweig, Germany) as described and validated previously [22].

Statistical Analysis

All values are expressed as mean ± SEM. Two-way repeated-measures ANOVA was used to detect differences within each experimental group. For comparison between homozygous male TGRs and male HanSDs, repeated-measures ANOVA was used with a test of interaction to determine whether the changes observed with experimental manipulations (diet manipulation and pharmacological treatment) were different between TGRs and HanSDs. One-way ANOVA was used for heart and kidney weights and for ET-1 concentrations and also for glomerulosclerosis data. Statistical significance was defined as $p < 0.05$.

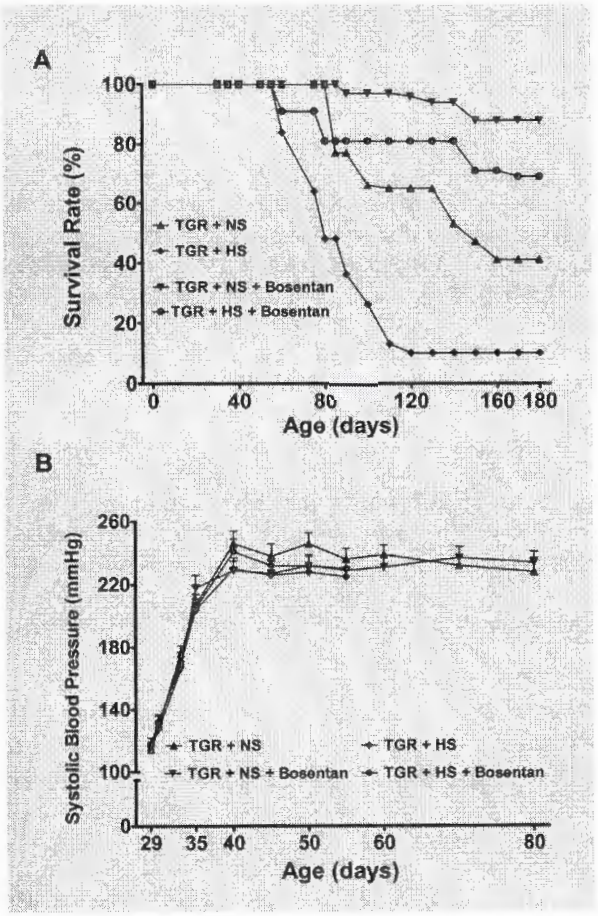


Fig. 1. Survival rate (**A**) and changes in systolic blood pressure (**B**) for homozygous male Ren-2 transgenic rats (TGR) fed either a normal (NS) or high salt (HS).

Results

Survival Rate

All HanSDs fed either the NS or HS diet with or without bosentan treatment survived until the end of the experiments (180 days of age). Some of the untreated homozygous male TGRs on the NS diet died starting at 82 days of age, and the final survival rate was 41% (14 of 34 rats). The HS diet in homozygous male TGRs accelerated the onset of the malignant phase of hypertension, thus the animals began to die at the age of 58 days, and the survival rate was only 10% (3 of 31 rats; $p < 0.05$ versus NS diet). Treatment with bosentan improved the survival rate in homozygous male TGRs fed the NS or HS diet to 88% (28 of 32 rats) and 69% (22 of 32 rats), respectively ($p < 0.05$ versus untreated; fig. 1A).

Blood Pressure

SBP determined by tail plethysmography was similar in conscious male HanSDs on the NS and HS diets and remained within the normotensive range throughout the whole experiment. The final SBP in HanSDs fed the NS and HS diets was 138 ± 5 and 128 ± 6 mm Hg, respectively. Treatment with bosentan did not significantly alter SBP in male HanSDs fed the HS or NS diet (data are not shown). There were also no significant differences in MAP measured intra-arterially in anesthetized rats on day 180 of age (table 2).

Neither the HS diet nor treatment with bosentan altered the rapid onset of hypertension in homozygous male TGRs. SBP data are shown to the time point when the survival rate in male homozygous TGRs was still 100% (fig. 1B).

Body and Organ Weights and Renal Histology

The course of BW gain in male HanSDs (fig. 2A) was not altered either by dietary manipulation or by treatment with bosentan. As shown in figure 2B, homozygous male TGRs fed either the NS or HS diet had an impaired BW gain compared with male HanSDs. Treatment with bosentan markedly improved the course of BW gain in homozygous male TGRs (all data are again shown at the time point when each group had a 100% survival rate).

Indices of HW/BW and KW/BW are summarized in table 2. Neither dietary manipulations nor treatment with bosentan significantly altered the HW/BW index in male HanSDs. As expected, homozygous male TGRs on the NS diet exhibited a higher HW/BW index than male HanSDs fed the NS diet. Treatment with bosentan significantly reduced the HW/BW index in homozygous male TGRs fed the NS diet. TGRs exposed to the HS diet had a significantly higher HW/BW index compared with NS-fed littermates. Administration of bosentan in homozygous TGRs fed the HS diet lowered the HW/BW index to the levels observed in NS-fed animals. Salt intake or bosentan treatment had no significant effects on the KW/BW index in all groups.

As shown in table 2, homozygous male TGRs fed the NS diet exhibited markedly higher glomerulosclerosis indices compared with male HanSDs which were further pronounced with the HS diet. Bosentan treatment substantially reduced the glomerulosclerosis index in homozygous male TGRs on the NS and HS intake.

HanSDs fed either the NS or the HS diet had a normal histological structure of the renal parenchyma. As shown in table 2, homozygous male TGRs fed the NS diet exhibited markedly higher glomerulosclerosis indices compared with male HanSDs, which was further pronounced with the HS diet. Bosentan substantially reduced the glomerulosclerosis index in homozygous TGRs on NS and HS intake. In addition, homozygous male TGRs fed the NS diet exhibited marked focal atrophy of the renal parenchyma, mostly localized in the inner cortex with the interstitium widened in areas with tubular atrophy. Large arteries showed fibrous intimal thickening that resulted in a reduction in the vessel lumen. All these changes were more pronounced in TGRs fed the HS diet which, in addition, led to fibrinoid necrosis in small interlobular and pre-glomerular arteries. Bosentan treatment markedly attenuated all these changes (fig. 3).

Proteinuria, Clearance of Endogenous Creatinine and Urinary Electrolyte Excretion

HanSDs fed either the NS or the HS diet exhibited minimal proteinuria and stable renal function (measured as clearance of endogenous creatinine) throughout the

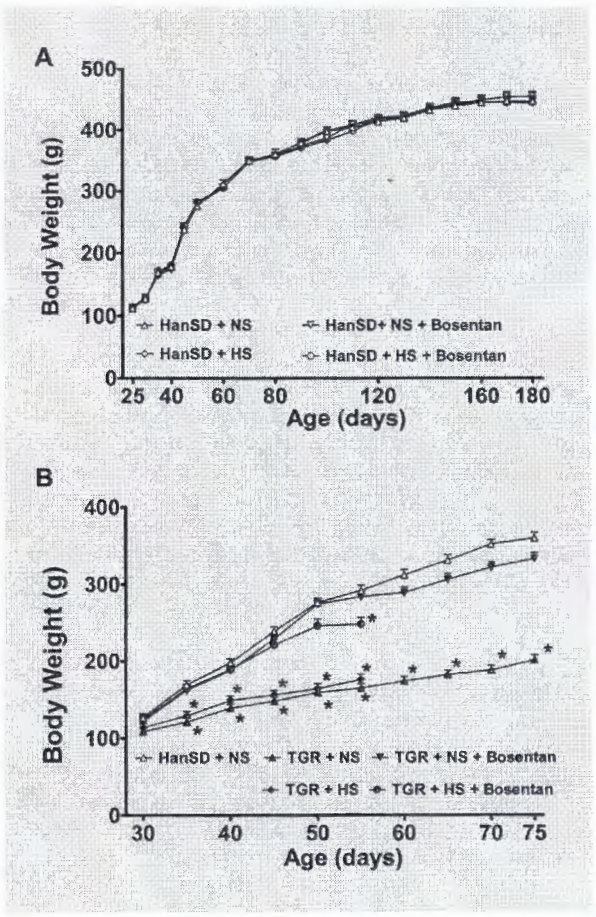


Fig. 2. Changes in body weight in transgene-negative (HanSD) (**A**) and homozygous male Ren-2 transgenic rats (TGR) (**B**) fed either a normal-salt (NS) or high-salt (HS) diet. * $p < 0.05$ vs. unmarked values at the same time point.

Table 2. Mean arterial pressure, heart and kidney weights, and glomerulosclerosis index in experimental groups at 180 days of age

Group	MAP, mm Hg	HW/BW, mg/g	KW/BW, mg/g	GI
HanSD + NS	117±4	3.12±0.19	3.29±0.17	0.33±0.21
HanSD + NS + bosentan	119±4	3.11±0.14	3.33±0.14	0.43±0.20
HanSD + HS	116±5	3.08±0.11	3.22±0.15	0.21±0.20
HanSD + HS + bosentan	121±6	3.09±0.10	3.31±0.16	0.40±0.25
TGR + NS	214±9[a]	4.18±0.12[a]	3.36±0.24	2.75±0.25[a]
TGR + NS + bosentan	209±10[a]	3.67±0.19[a, b]	3.39±0.22	1.58±0.20[a, b]
TGR + HS	210±8[a]	4.85±0.24[a, c]	3.31±0.18	3.00±0.00[a, c]
TGR + HS + bosentan	212±11[a]	4.21±0.19[a, b]	3.28±0.21	1.62±0.20[a, b]

HanSD = Transgene-negative rats; TGR = homozygous transgenic rats, (mRen2)27; NS = normal-salt diet; HS = high-salt diet; MAP = mean arterial pressure; HW = heart weight; BW = body weight; KW = kidney weight; GI = glomerulosclerosis index.

[a] $p < 0.05$ vs. HanSD rats; [b] $p < 0.05$ bosentan-treated vs. bosentan-untreated; [c] $p < 0.05$ vs. all other values.

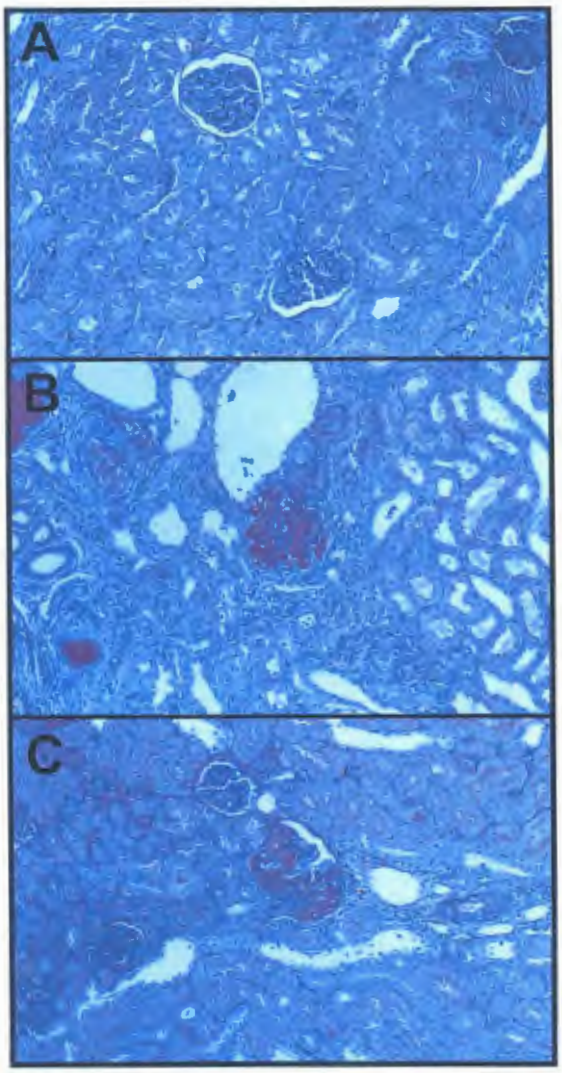


Fig. 3. Representative histological slides of kidneys from transgene-negative (HanSD) and homozygous male Ren-2 transgenic rats (TGR). × 10. **A** HanSD rats fed the normal (NS) diet reveal a normal renal parenchyma. **B** TGR fed the NS diet exhibit marked atrophy of the renal parenchyma and hyalinized glomeruli (center) and a sclerotic one (upper left). Around the hyalinized glomeruli, tubular loss with interstitial fibrosis can be seen. The afferent arteriole shows mucinous intimal thickening. **C** TGR fed the NS diet and treated with bosentan exhibit only moderate glomerulosclerosis and hyalinized glomeruli (center).

entire experimental period. Bosentan treatment did not affect these parameters in any of the experimental groups (table 3).

As shown in figure 4, homozygous male TGRs fed the NS diet developed strong proteinuria and the HS diet further accelerated the degree of proteinuria. Bosentan treatment markedly ameliorated the development of proteinuria in homozygous male TGRs fed the NS or the HS diet, but did not fully prevent the development of proteinuria (fig. 4). Data on proteinuria obtained from all experimental groups throughout the duration of the study are shown in table 3.

We observed significant decreases in the clearance of endogenous creatinine in all animals at the time before the individual animals died, but thereafter in survivors the clearance of endogenous creatinine returned to levels observed in control animals. In table 3, only the data from animals that survived until the end of the experiment are presented.

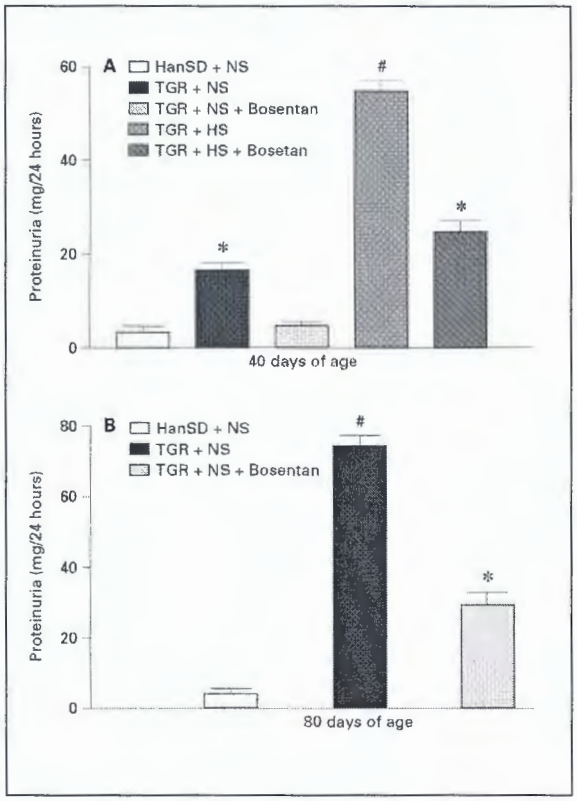
Animals fed the HS diet exhibited significantly higher daily urinary sodium excretion compared with rats fed the NS diet (between 1,640 and 1,820 versus 820 and 880 µmol/24 h, $p < 0.05$).

ET-1 Tissue Concentrations

As shown in figure 5A, kidney cortex ET-1 concentrations in male HanSDs were not different from those in homozygous male TGRs on the NS diet (0.14 ± 0.03 and 0.20 ± 0.02 fmol/mg protein, respectively). The HS diet increased moderately but not significantly the kidney cortex ET-1 levels in HanSDs and TGRs (0.20 ± 0.03 and 0.33 ± 0.07 fmol/mg protein, respectively). Likewise, the left heart ventricular ET-1 concentrations were not significantly different between HanSDs and TGRs fed the NS diet (0.53 ± 0.06 and 0.58 ± 0.04 fmol/mg protein, respectively) but were significantly higher in TGRs fed the HS diet (0.51 ± 0.06 vs. 1.72 ± 0.18 fmol/mg protein, $p < 0.05$; fig. 5B).

Discussion

The first aim of the present study was to examine the effects of treatment with bosentan on the course of malignant hypertension in homozygous TGRs. We found that administration of bosentan markedly improved the survival rate in homozygous male TGRs without a significant reduction in BP. In addition, bosentan treatment ameliorated end-organ damage in homozygous TGRs, i.e. it reduced cardiac hypertrophy, diminished proteinuria



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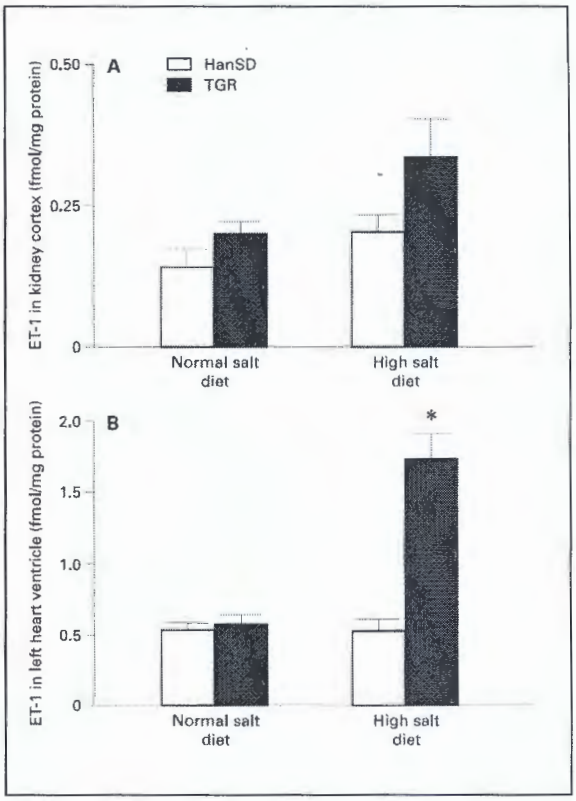

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Fig. 4. Comparison of proteinuria between transgene-negative (HanSD) and homozygous male Ren-2 transgenic rats (TGR) fed either a normal-salt (NS) or high-salt (HS) diet either at age 40 days (**A**) or at age 80 days (**B**). * $p < 0.05$ vs. unmarked values. # $p < 0.05$ vs. all other values.

Fig. 5. Kidney cortex (**A**) and left ventricular heart (**B**) ET-1 levels in transgene-negative (HanSD) and homozygous male Ren-2 transgenic rats (TGR). * $p < 0.05$ vs. unmarked values.

Table 3. Proteinuria and clearance of endogenous creatinine throughout the whole experiment

Group	Day 40		Day 80		Day 120		Day 170	
	proteinuria mg/24 h	C_{Cr} µl/min/g	proteinuria mg/24 h	C_{Cr} µl/min/g	proteinuria mg/24 h	C_{Cr} µl/min/g	proteinuria mg/24 h	C_{Cr} µl/min/g
HanSD + NS	3.1±1.1	7.7±0.6	4.1±1.2	7.6±0.5	5.2±1.1	7.2±0.4	5.4±1.3	7.8±1.4
HanSD + NS + bosentan	2.9±0.9	7.6±0.5	3.6±1.1	7.8±0.7	4.9±1.2	7.3±0.5	5.3±1.2	7.6±1.1
HanSD + HS	6.4±1.3	6.8±0.7	7.7±1.5	7.1±0.4	6.2±1.1	7.1±0.6	7.8±1.1	7.0±0.9
HanSD + HS + bosentan	5.4±1.2	7.2±0.6	5.4±1.3	6.6±0.5	5.7±0.8	7.6±0.5	7.7±1.2	7.1±0.8
TGR + NS	14.9±1.2[a]	6.6±0.7	66.4±3.1[a]	6.2±0.5	61.7±3.9[a]	6.2±0.8	41.7±4.7[a]	6.8±0.8
TGR + NS + bosentan	4.2±1.1[b]	6.9±0.6	22.1±2.9[a, b]	6.9±0.7	26.5±3.2[a, b]	6.8±0.7	21.7±2.8[a, b]	6.6±1.0
TGR + HS	49.3±2.2[a, b]	6.3±0.4	45.6±4.4[a]	6.5±0.5	48.1±4.9[a]	6.3±0.8	50.3±1.8[a]	6.7±0.8
TGR + HS + bosentan	22.1±2.1[a, b]	6.7±0.7	20.4±3.9[a, b]	6.8±0.7	19.1±4.2[a, b]	6.4±0.7	20.1±4.3[a, b]	6.4±0.7

HanSD = Transgene-negative rats; TGR = homozygous transgenic rats, (mREn2)27; NS = normal-salt diet; HS = high-salt diet; C_{Cr} = clearance of endogenous creatinine expressed per gram of body weight.

[a] $p < 0.05$ vs. HanSD rats; [b] $p < 0.05$ bosentan-treated vs. bosentan-untreated TGR.

and decreased the glomerulosclerosis index. These results are consistent with results from previous studies in transgenic rats harboring both human renin and angiotensinogen genes (dTGR) which showed that either ET receptor blockade or ET-converting enzyme inhibition reduced the mortality rate and ameliorated end-organ damage independent of BP changes [12, 23, 24].

The second aim of this study was to assess the effects of the HS diet on the course of hypertension and associated end-organ damage in homozygous male TGRs. With respect to BP, we found that HS intake did not accelerate the development of hypertension in homozygous male TGRs. However, the HS diet substantially worsened the mortality rate and deteriorated the cardiac and renal injury in these animals. ET receptor blockade resulted in a considerable improvement in survival rate and a substantial attenuation of end-organ damage in homozygous male TGRs fed the HS diet without influencing BP levels.

Taken together, our data indicate that an ET-dependent component plays a critical role in the development of end-organ damage in homozygous male TGRs. Our findings are consistent with results from previous studies in other ANG II-dependent models of hypertension and further support the hypothesis that ET receptors contribute strongly to ANG II-induced end-organ damage [8–12, 23, 24; for review, see 2, 4].

Nevertheless it is important to mention that in some previous studies it has been demonstrated that ET blockade did not lower BP and did not prevent end-organ damage in heterozygous male TGRs despite enhanced preproET mRNA expression and ET-1 concentration in renal and cardiac tissues, suggesting that an ET-dependent component is negligible in this model of hypertension [25–27]. We cannot offer a fully satisfying explanation for the discrepancy between our and previous results, but one critical difference should be considered. In studies showing a minor ET-dependent component in the development of hypertension and end-organ damage, heterozygous male TGRs were used whereas in our study homozygous male TGRs were employed [25–27]. It is known that homozygous male TGRs exhibit a very early onset of the malignant phase of hypertension (characterized by body weight loss, increased proteinuria and high mortality rate) and that the transition to the malignant phase arises in heterozygous male TGRs at a later time [15, 28; for review, see 16]. In addition, it has been shown that the differences in genetic background among TGR colonies alter the phenotype in this model (especially the incidence of the malignant phase of hypertension) [29]. Taken together, it seems reasonable to assume that the differences between homozygous and heterozygous male TGRs might be responsible for the discrepancies regarding the effects of ET blockade on the development of end-organ damage in this model.

The lack of a BP-lowering effect of bosentan in TGRs cannot be ascribed to insufficient ET receptor blockade since the dose of bosentan employed in the present study was sufficient in our pilot studies to prevent BP responses to ET-1 (250 ng i.v.) in TGRs as well as in HanSDs. With this respect, the nonselective nature of this compound should be considered. An increasing body of evidence indicates that the hypertensinogenic actions of ET-1 are produced via ET_A receptor activation [8, 30; for review, see 2]. In addition, Vassileva et al. [31] recently demonstrated that ET-mediated activation of ET_B receptors participates in the pressure-natriuresis mechanism; it has been proposed that ET may participate via this mechanism in the long-term regulation of BP. Therefore, it seems reasonable to assume that selective ET_A blockade would lower BP in TGRs. However, recent results from Seccia et al. [26] do not support this notion. They found that BMS-182874 treatment (a selective ET_A receptor antagonist) did not lower BP in TGRs. Moreover, the concept that ET_A receptor activation leads to vasoconstriction and ET_B receptor stimulation causes vasodilatation [for review, see 2] may be oversimplified because it is now known that two distinct subtypes of ET_B receptors exist (ET_{B1} and ET_{B2}). ET_{B1} receptors are present on the vascular endothelium and cause vasodilatation through the release of nitric oxide and prostaglandins [32], whereas ET_{B2} receptors are found on vascular smooth muscle cells and mediate non-ET_A vasoconstriction [33]. In addition, in their comprehensive study Just et al. [34] have recently shown that the selective activation of ET_B receptors mediates net renal vasoconstriction. Furthermore, ET_B receptors have been reported to stimulate aldosterone production [for review, see 35] and recently it has been shown that aldosterone plays an important role in the development of cardiac and renal vascular fibrosis [25, 36, 37]. Therefore, to evaluate the overall role of the ET system in the development of ANG II-dependent hypertension, it seems that nonselective ET_A/ET_B receptor blockade is a suitable experimental approach.

It is obvious, however, that more detailed studies employing selective ET receptor antagonists are needed to address the role of specific ET receptor subtypes in the development of hypertension in TGRs.

The most important issue of the present study is related to the question: what are the underlying mechanisms (or mechanism) responsible for the beneficial effects of

ET blockade on survival rate and end-organ damage in homozygous male TGRs fed the NS or HS diet?

Multiple lines of independent evidence indicate that these beneficial effects of ET blockade are BP-independent. It has been shown that antihypertensive treatment with hydralazine, reserpine and hydrochlorothiazide, which decreased BP to normotensive levels, barely delayed end-organ damage in dTGRs, whereas treatment with bosentan markedly ameliorated it [38]. In addition, it has been demonstrated in 2K1C Goldblatt hypertensive rats that chronic ET_A receptor blockade attenuated cardiac hypertrophy [13] and ameliorated the development of myocardial fibrosis [14] without a reduction in BP. Moreover, two studies reported that mice overexpressing ET-1 developed pronounced end-organ damage independent of an increase in systemic BP [39, 40]. In this regard it is important to note that in our study we found increased left ventricular ET-1 concentrations in homozygous male TGRs fed the HS diet when compared with male HanSDs, which may contribute to the development of end-organ damage. However, this issue requires further studies.

With respect to the underlying mechanism(s), it has been found in these rats that bosentan administration inhibited the activation of the nuclear factor-kappa B (NF-κB) and of the transcription factor activator protein (AP)-1 in the kidney and in the heart independent of BP [12]. Based on a previous report that NF-κB activation has an impact on renal disease progression [41], it has been proposed that one possible mechanism for the beneficial effects of ET blockade on the development of end-organ damage might be to block the activation of the NF-κB signal transduction pathway [42].

With these observations in mind, the potential role of an interaction between the RAS and the ET system became apparent and received increasing attention. Thus, Bohlender et al. [23] reported that the combined blockade of ET and ANG II receptors had synergistic effects on the survival rate and protection from end-organ damage in dTGRs. With respect to the role of the RAS, it has been demonstrated that hypertensive TGRs more often have manifest cardiovascular end-organ damage when compared with age- and BP-matched spontaneously hypertensive rats (SHRs) [43] and that blockade of the RAS prevented end-organ damage without a reduction in BP in TGRs [44, 45]. Furthermore, mice lacking the cardiac and renal angiotensinogen gene exhibited reduced end-organ damage compared with wild-type control animals [46]. Taken together, these findings corroborate that tissue activity of the RAS is a major determinant of hypertension-induced end-organ damage [for review, see 3].

Alternatively, a second possible mechanism contributing to the beneficial effects of ET blockade might be related to the blockade of aldosterone-mediated deleterious actions on organ structure. It has recently been shown that ET-1 plays a critical role in the development of end-organ damage in aldosterone-infused rats exposed to HS intake and that ET blockade prevented cardiac and aortic fibrosis independent of BP changes [35]. In addition, it has been reported that ET-1 exerts an important permissive action on basal aldosterone secretion and that treatment with bosentan decreased basal aldosterone secretion in TGRs [25]. This notion is further supported by recent findings that ET receptor blockade in aldosterone-infused rats decreased oxidative stress [37]. Since it is well known that oxidative stress increases the activity of NF-κB [for review, see 47] and that oxidative stress plays an important role in ANG II-dependent models of hypertension [for review, see 48], it seems conceivable that the beneficial effects of bosentan treatment may be attributed to the blockade of the aldosterone-mediated deleterious effects on organ structure.

In summary, in the present study we showed that treatment with bosentan dramatically improved the survival rate in homozygous TGRs fed a NS or HS diet in the absence of a BP reduction. Administration of bosentan in these TGRs also markedly decreased cardiac hypertrophy and renal damage, thus providing considerable protection from end-organ damage.

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II.A Nejdůležitější závěry experimentální studie

V naší první experimentální studii bylo zjištěno, že blokáda endotelinových receptorů u Ren2 transgenních potkanů TGR(mRen2)27 výrazným způsobem snižuje mortalitu experimentálních zvířat s maligní hypertenzí, aniž by byl signifikantně ovlivněn arteriální krevní tlak.

Příčina nesignifikantního snížení krevního tlaku u homozygotních transgenních potkanů je pravděpodobně v charakteru neselektivní blokády ET_A a ET_B receptorů a jejich nerovnoměrném rozmístění v jednotlivých orgánových systémech.

Aktivace ET_A receptorů vede k vazokonstrikci, aktivace ET_{B1} receptorů působí přes NO a prostaglandiny naopak vazodilataci a ET_{B2} receptory, které se nacházejí především na hladkých svalových buňkách cév, pak po aktivaci způsobí vazokonstrikci.

Dále pak aktivace ET_B receptorů stimuluje produkci aldosteronu, který má důležitou úlohu v rozvoji srdeční a renální aterosklerózy.

Jako druhý cíl studie pak naše studie prokázala, že vysokoslaná dieta neakceleruje rozvoj hypertenze u homozygotních transgenních potkanů, ale podílí se na významném zhoršení mortality a orgánového poškození srdce a ledvin

II. B Chronická blokáda endotelinových receptorů redukuje hypertenzní orgánové postižení u heterozygotních Ren-2 transgenních potkanů na vysokoslané dietě, aniž by ovlivňovala arteriální krevní tlak

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Chronic Endothelin Receptor Blockade Reduces End-Organ Damage Independently of Blood Pressure Effects in Salt-Loaded Heterozygous Ren-2 Transgenic Rats

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Summary

The present study was performed to evaluate the role of an interaction between the endothelin (ET) and the renin-angiotensin systems (RAS) in the development and maintenance of hypertension and in hypertension-associated end-organ damage in heterozygous male and female transgenic rats harboring the mouse Ren-2 renin gene (TGR). Twenty-eight days old heterozygous TGR and age-matched transgene-negative normotensive Hannover Sprague-Dawley rats (HanSD) were randomly assigned to groups with normal-salt (NS) or high-salt (HS) intake. Nonselective ET_A/ET_B receptor blockade was achieved with bosentan (100 $mg.kg^{-1}.day^{-1}$). All male and female HanSD as well as heterozygous TGR on NS exhibited 100 % survival rate until 180 days of age (end of experiment). HS diet in heterozygous TGR induced a transition from benign to malignant phase hypertension. The survival rates in male and in female heterozygous TGR on the HS diet were 46 % and 80 %, respectively, and were significantly improved by administration of bosentan to 76 % and 97 %, respectively. Treatment with bosentan did not influence either the course of hypertension (measured by plethysmography in conscious animals) or the final levels of blood pressure (measured by a direct method in anesthetized rats) in any of the experimental groups of HanSD or TGR. Administration of bosentan in heterozygous TGR fed the HS diet markedly reduced proteinuria, glomerulosclerosis and attenuated the development of cardiac hypertrophy compared with untreated TGR. Our data show that the ET receptor blockade markedly improves the survival rate and ameliorates end-organ damage in heterozygous TGR exposed to HS diet. These findings indicate that the interaction between the RAS and ET systems plays an important role in the development of hypertension-associated end-organ damage in TGR exposed to salt-loading.

Key words

Hypertension • Endothelin system • Renin-angiotensin system • Bosentan • End-organ damage

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Introduction

Growing body of evidence indicates that the endothelin (ET) system plays an important role in the pathogenesis of hypertension and associated end-organ damage in angiotensin II (ANG II)-dependent models induced by exogenously administrated ANG II, but not in the models with enhanced endogenous production of ANG II (d´Uscio *et al.* 1997, Ehmke *et al.* 1999, Müller *et al.* 2000, Alexander *et al.* 2001, Ficai *et al.* 2001, Müller *et al.* 2002, Sasser *et al.* 2002, Saam *et al.* 2003, for review see Moreau and Schiffrin 2003). The reasons for this discrepancy regarding the role of ET system in the ANG II-dependent model of hypertension remain unclear.

To determine the contribution of an interaction between endothelin-1 (ET-1) and ANG II to the development of hypertension and related end-organ damage in a model of hypertension dependent on the endogenous activation of the renin-angiotensin system (RAS), we utilized the rat strain transgenic for mouse Ren-2 renin gene [TGR; strain name TGR (mRen2)27]. The development of hypertension in this strain is a result of insertion of the mouse Ren-2 renin gene into the rat genome (Mullins *et al.* 1990). Thus, TGR represents a model of hypertension with a well-defined genetic background, in which the development of hypertension can be attributed to a single gene alteration and is clearly ANG II-dependent (for review see Langheinrich *et al.* 1996). Being characterized by normal plasma ANG II and by overexpression of the Ren-2 transgene in the adrenal cortex and in the arterial wall, the TGR seems to be an optimal model of ANG II-dependent hypertension with endogenous activation of the RAS (Hilgers *et al.* 1992, Peters *et al.* 1993, Jacinto *et al.* 1999, for review see Langheinrich *et al.* 1996).

However, only limited and conflicting data regarding the role of the ET system in TGR are available at present. On one hand, it has been reported that ET blockade resulted in hypotensive effects in male and female heterozygous TGR pointing to an important ET-dependent component in this model of hypertension (Gardiner *et al.* 1995, 2000, Kelly *et al.* 2000). In addition, we have recently showed that nonselective ET_A/ET_B receptor blockade markedly improves the survival rate and ameliorates end-organ damage in homozygous male TGR without a significant lowering of blood pressure (BP) (Dvořák *et al.* 2002a,b). On the other hand, it has been demonstrated that ET receptor blockade did not lower BP and did not prevent end-organ damage in heterozygous male TGR despite increased preproendothelin mRNA expression and ET-1 concentration in renal and cardiac tissues suggesting that an ET-dependent component is negligible in this model of hypertension (Whitworth *et al.* 1995, Andreis *et al.* 2000, Rossi *et al.* 2000, Seccia *et al.* 2003). Because the variance of these findings may have resulted from different time protocols and from a different ET blocker used, the rationale of the present study was to elucidate the role of ET system in the development of hypertension in heterozygous TGR under precisely defined experimental conditions (both sexes and the long-term follow-up).

Accordingly, the first aim of the present study was to examine the effects of nonselective ET_A/ET_B receptor blockade by bosentan on the course of hypertension in heterozygous male TGR. Since it has been reported that TGR also show a salt-sensitive component of hypertension (Callahan *et al.* 1996), and we have demonstrated that high-salt diet augmented the onset of the malignant phase of hypertension in homozygous male TGR (Dvořák *et al.* 2002a,b), the second aim of this study was to delineate whether a high-salt diet would accelerate the course of hypertension and end-organ damage in heterozygous TGR. Finally, since an emerging body of evidence indicates that TGR exhibit a marked sexual dimorphism with respect to the course of hypertension (Lee *et al.* 1996, Cargnelli *et al.* 1998) and that the ET system also reveals noticeable gender differences in the intrarenal activity (Taylor *et al.* 2003), the third aim of the present study was to compare the effects of salt intake and pharmacological manipulation on the cardiovascular phenotypes in heterozygous male and female TGR.

Methods

Protocols in the present study were designed according to the Guiding Principles in the Care and Use of Animals approved by the Council of the American Physiological Society and were approved by the Czech Animal Care and Use Committee (protocol 79#2001).

Animals

All animals used in the present study were bred at the Center for Experimental Cardiovascular Research of the Institute for Clinical and Experimental Medicine from stock animals supplied from the Max Delbrück

Center for Molecular Medicine in Berlin, Germany. Animals were kept on a 12-hour/12-hour light/dark cycle.

Diets

All diets used in the present study were produced by SEMED (Prague, Czech Republic). Rats were fed either a normal-salt diet (NS, 0.45 % NaCl, 19-21 % protein) or a high-salt diet (HS, 2 % NaCl, 19-21 % protein).

Experimental design and functional examination

Twenty-eight days old heterozygous male and female TGR and age-matched transgene-negative Hannover Sprague-Dawley (HanSD) male and female rats from several litters were randomly assigned to experimental groups with care that animals from a single litter did not prevail in any of the groups. Food intake was monitored once a week and the concentration of bosentan (Actelion, Switzerland) was added to the NS and HS diets in a concentration depending on the food intake so that the final consumption of bosentan was 100 $mg.kg^{-1}$ of body weight per day. For this purpose, new diets were prepared every week. The experimental groups with initial number of animals are shown in Table 1.

Table 1. Mean arterial pressure, heart and kidney weights, and glomerulosclerosis index (GI) in experimental groups at 180 days of age.

Group	n	MAP (mm Hg)	HW/BW (mg/g)	KW/BW (mg/g)	GI
Male HanSD + NS	13	117±4	3.12±0.19	3.29±0.17	0.33±0.21
Male HanSD + NS + Bosentan	14	119±4	3.11±0.14	3.33±0.14	0.43±0.20
Male HanSD + HS	14	116±5	3.08±0.11	3.22±0.15	0.21±0.20
Male HanSD + HS + Bosentan	14	121±6	3.09±0.10	3.31±0.16	0.40±0.25
Female HanSD + NS	16	109±6	3.12±0.14	3.27±0.19	0.28±0.19
Female HanSD + NS + Bosentan	15	112±6	3.14±0.17	3.22±0.12	0.33±0.24
Female HanSD + HS	14	113±5	3.11±0.09	3.41±0.20	0.41±0.22
Female HanSD + HS + Bosentan	15	114±4	3.08±0.12	3.21±0.17	0.37±0.26
Male TGR + NS	24	199±7*#	3.82±0.27*	3.36±0.19	1.44±0.18*
Male TGR + NS + Bosentan	26	192±6*#	3.84±0.22*	3.23±0.21	1.68±0.13*
Male TGR + HS	22	191±9*#	4.49±0.28*+	3.29±0.19	2.24±0.12*+
Male TGR + HS + Bosentan	25	193±8*#	3.78±0.25*	3.35±0.18	1.60±0.16*
Female TGR + NS	32	158±6*	3.68±0.19*	3.32±0.16	1.51±0.15*
Female TGR + NS + Bosentan	24	159±5*	3.63±0.21*	3.31±0.21	1.43±0.16*
Female TGR + HS	35	157±8*	4.27±0.23*+	3.20±0.13	2.33±0.14*+
Female TGR + HS + Bosentan	34	158±9*	3.67±0.20*	3.25±0.19	1.39±0.13*

HanSD, transgene-negative rats; TGR, heterozygous transgenic rats (mRen2)27; NS, normal salt diet; HS, high salt diet; MAP, mean arterial pressure; HW, heart weight; BW, body weight; KW, kidney weight. * P<0.05 vs. HanSD rats; # P<0.05 male TGR vs. female TGR; + P<0.05 untreated TGR fed HS vs. TGR fed NS or HS and treated with bosentan

Systolic blood pressure (SBP) was measured in conscious animals by tail plethysmography from 29 to 40 days of age every two days, from 41 to 60 days of age every three days and thereafter every week until the end of the experiment (180 days of age). At each measurement, BP was determined as the mean of four measurements. This method was previously validated in our laboratory by Heller and Hellerová (1998). At 40, 80, 120 and 170 days of age, rats were placed into individual metabolic cages and their 24-hour urine was collected (after at least 3 days of training period) for protein determination and calculation of clearance of endogenous creatinine (a blood sample for determination of plasma concentration of creatinine and electrolytes was taken on the second day in the morning). Body weights were obtained during SBP measurements and from day 60 until

the end of the experiment rats were weighed additionally twice a week. At the end of experiments, animals were anesthetized with thiopental sodium (50 mg.kg^{-1} of body weight), and the right carotid artery was cannulated with PE-50 tubing for direct measurement of mean arterial pressure (MAP).

Morphological examination

The ratios of heart weight/body weight (mg/g) and right kidney weight/body weight (mg/g) were used as indices of cardiac hypertrophy (HW/BW) and kidney hypertrophy (KW/BW), respectively. The left kidney was quickly removed, fixed in 4 % formaldehyde, dehydrated and embedded. Paraffin sections were stained with hematoxylin-eosin and periodic acid-Schiff reaction (PAS). Slides were evaluated in a blind fashion. Fifty glomeruli in each kidney were examined on a semi-quantitative scale as described previously (Yagil *et al.* 2002): *grade 0*, all glomeruli normal; *grade 1*, one to two glomeruli were affected; *grade 2*, more than 2 but less than 17 glomeruli were affected; *grade 3*, 17 or more glomeruli were affected.

Tissue ET-1 evaluation

In separate groups of HanSD and heterozygous TGR fed either NS or HS diet (n = 8 in each group) from 28 to 80 days, ET-1 concentrations were examined in kidney cortex and left ventricle. The purpose of this protocol was to evaluate whether tissue ET-1 levels are increased before transition to the malignant phase of hypertension in this model. This time protocol was chosen because at this time point (80 days of age) each group still exhibited 100 % survival rate. ET-1 levels were assessed by the ELISA system from Amersham (Braunschweig, Germany) as described and validated previously (Bäcker *et al.* 2001).

Statistical analysis

All values are expressed as mean ± S.E.M. Two-way repeated-measures ANOVA were used to detect differences within each experimental group. For comparison between heterozygous TGR and HanSD rats, repeated-measures ANOVA was used with a test of interaction to determine whether the average change after experimental manipulation (diet manipulation and pharmacological treatment) was different between TGR and HanSD rats. One-way ANOVA was used for the evaluation of heart and kidney weights, ET-1 concentrations and glomerulosclerosis data. Statistical significance was defined as $p<0.05$.

Results

Survival rate

All male and female HanSD fed either the NS or HS diet with or without bosentan treatment survived until the end of the experiments (180 days of age). Likewise all heterozygous male and female TGR fed NS diet with or without bosentan administration exhibited 100 % survival rate. In contrast, only 46 % (10 of 22 rats) heterozygous male TGR exposed to HS intake survived until the end of the experiment (Fig. 1A). The administration of bosentan significantly increased the survival rate in this group to 76 % (19 of 25 rats). Likewise, HS diet in heterozygous female TGR resulted in the survival rate of 80 % (28 of 35 rats) which was significantly higher than that of heterozygous male TGR ($p<0.05$). This was improved by treatment with bosentan to 97 % (33 of 34 rats) ($p<0.05$) (Fig. 1B).

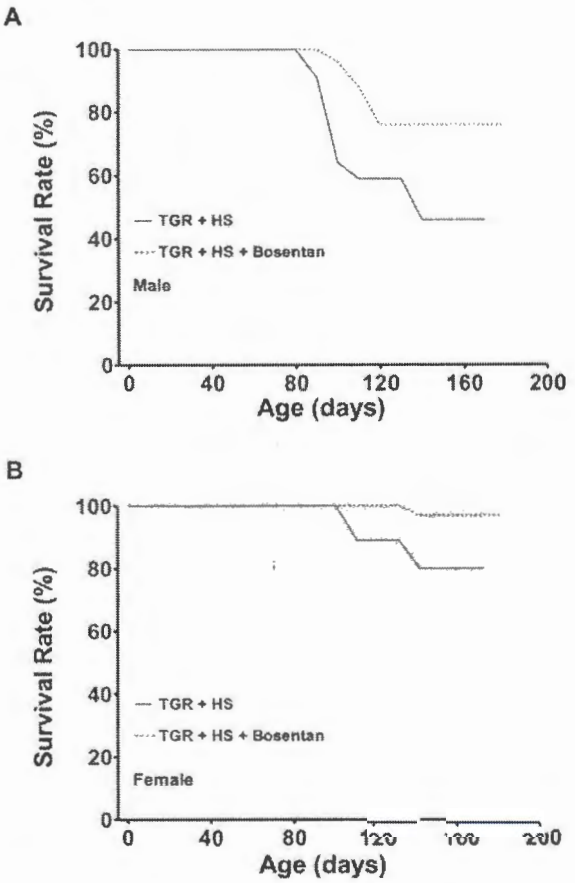

Fig. 1. Survival rate of male (A) and female (B) heterozygous Ren-2 transgenic rats (TGR) fed high salt (HS) diet.

Blood pressure

SBP determined by tail plethysmography in conscious male and female HanSD on NS diet remained within the normotensive range throughout the whole experiment and the final SBP was not different between male and female rats (138±5 and 128±6 mm Hg, respectively). HS diet did not significantly increase SBP at any time point throughout the experiment either in male or female HanSD and the final SBP was not significantly higher compared with animals fed the NS diet (139±6 vs. 138±5 in males and 137±5 vs. 128±6 mm Hg in females, respectively). Treatment with bosentan did not significantly alter SBP either in male or female HanSD fed HS or NS diet (data not shown). There were also no significant differences in MAP measured intraarterially in anesthetized rats on day 180 (Table 1).

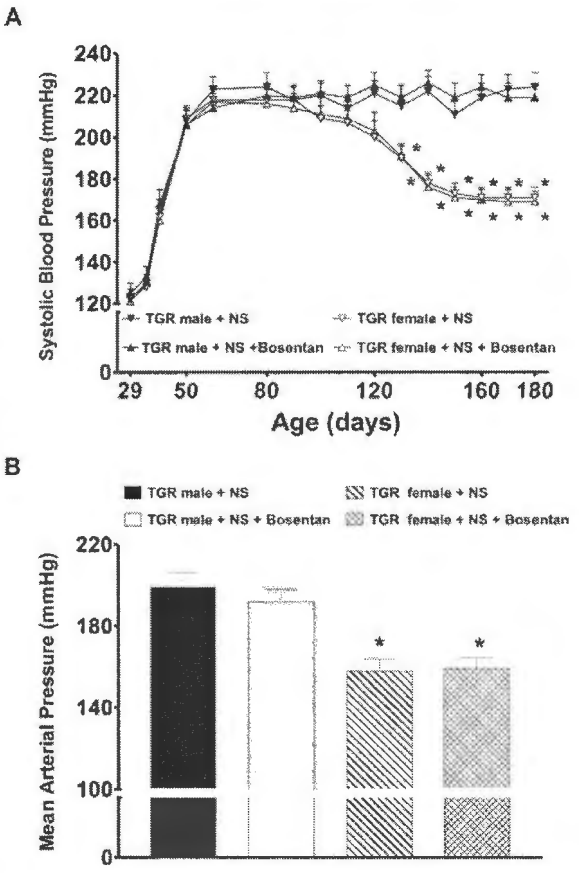

Fig. 2. Changes in systolic blood pressure in male and female heterozygous Ren-2 transgenic rats (TGR) fed normal (NS) diet (**A**). Mean arterial pressure measured at the end of experiment (180 days of age) (**B**).

As shown in Figure 2A, both heterozygous male and female TGR fed the NS diet developed hypertension at day 40 of age and reached the peak of BP at day 65 of age. In heterozygous male TGR fed the NS diet, SBP was stable until the end of the experiment. In contrast, in heterozygous female TGR fed the NS diet, SBP after reaching its peak (between 65 to 80 days of age), started to decrease spontaneously (at the age of 130 days) and the final SBP was significantly lower compared with male heterozygous TGR fed the NS diet. Treatment with bosentan did not alter this pattern of SBP in heterozygous male or female TGR fed the NS diet. Heterozygous female TGR fed the NS diet either untreated or treated with bosentan, had significantly lower MAP at the end of experiment than heterozygous male TGR fed the NS diet without or with bosentan (158±6 vs. 199±7 and 159±5 vs. 192±6 mm Hg, respectively, $p<0.05$ in both cases) (Fig. 2B).

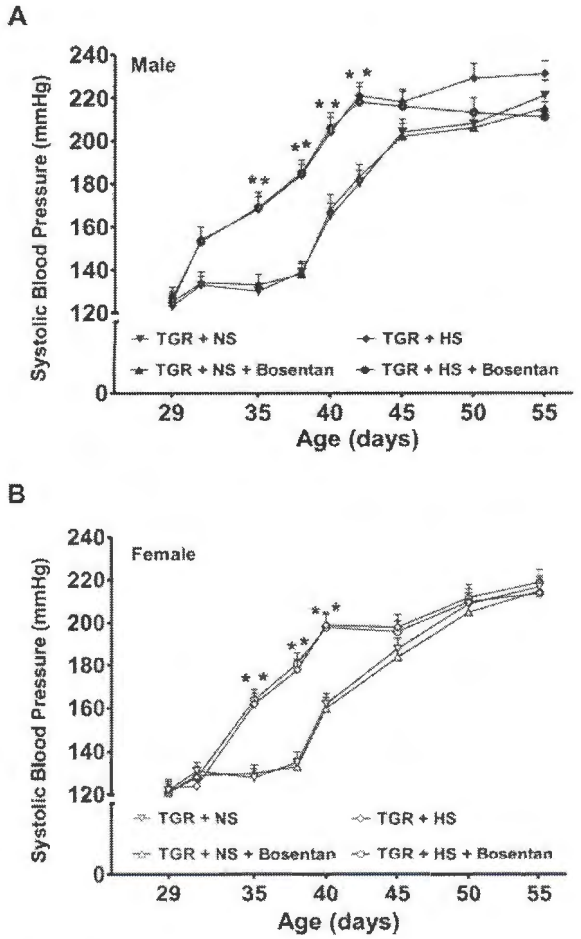

Fig. 3. Changes in systolic blood pressure in male TGR fed either NS or high salt (HS) diets (**A**). Changes in systolic blood pressure in female TGR fed either NS or HS diets (**B**). * $P<0.05$ vs. values at the same time point.

As shown in Figure 3A, the HS diet caused a significant acceleration of hypertension development in heterozygous male TGR between days 35 to 42 of age as compared with animals fed the NS diet and bosentan treatment did not prevent this acceleration. Likewise, between 35 to 40 days of age heterozygous female TGR fed the HS diet exhibited significantly higher SBP compared with animals fed the NS diet. Similarly, the administration of bosentan did not attenuate this BP increase (Fig. 3B).

Body and organ weights and glomerulosclerosis index

The course of body weight (BW) gain in male and female HanSD was not altered either by dietary manipulation or by the treatment with bosentan. The course of BW gain in heterozygous male and female TGR fed either the NS diet alone or the NS diet with bosentan was almost identical to that observed in HanSD animals. However, heterozygous male and female TGR exposed to HS diet exhibited a profound loss of BW just before they started to die (Figs 3A and 3B). In contrast, heterozygous male and female TGR fed the HS diet with bosentan showed the same BW gain as NS treated rats (data not shown)

Data on HW/BW and KW/BW are summarized in Table 1. As expected, heterozygous male as well as female TGR on NS diet exhibited a higher index of HW/BW compared with male and female HanSD fed the NS diet and treatment with bosentan did not alter this pattern. Heterozygous male as well as female TGR exposed to HS diet had a significantly higher HW/BW compared with NS fed littermates. Administration of bosentan in these groups of TGR lowered their HW/BW to levels observed in the animals fed NS diet. Neither diet manipulations nor treatment with bosentan did significantly change the HW/BW in male or female HanSD. There were no significant differences in the KW/BW index among the groups (Table 1).

As shown in Table 1, heterozygous male and female TGR fed the NS diet exhibited markedly higher values of glomerulosclerosis index compared with male and female HanSD. HS diet further increased the glomerulosclerosis index in these groups of TGR. Administration of bosentan in heterozygous male and female TGR fed the HS diet substantially reduced the glomerulosclerosis index to the levels observed in NS fed animals.

Table 2. Proteinuria, clearance of endogenous creatinine at different time points in male and female experimental groups.

Group	Day 40		Day 80		Day 120		Day 170	
	Proteinuria (mg/24 h)	C_{Cr} ($\mu l.min^{-1}.g^{-1}$)	Proteinuria (mg/24 h)	C_{Cr} ($\mu l.min^{-1}.g^{-1}$)	Proteinuria (mg/24 h)	C_{Cr} ($\mu l.min^{-1}.g^{-1}$)	Proteinuria (mg/24h)	C_{Cr} ($\mu l.min^{-1}.g^{-1}$)
Male HanSD + NS	3.1±1.1	7.7±0.6	4.1±1.2	7.6±0.5	5.2±1.1	7.2±0.4	5.4±1.3	7.8±1.4
Male HanSD + NS + B	2.9±0.9	7.6±0.5	3.6±1.1	7.8±0.7	4.9±1.2	7.3±0.5	5.3±1.2	7.6±1.1
Male HanSD + HS	6.4±1.3	6.8±0.7	7.7±1.5	7.1±0.4	6.2±1.1	7.1±0.6	7.8±1.1	7.0±0.9
Male HanSD + HS + B	5.4±1.2	7.2±0.6	54±1.3	6.6±0.5	5.7±0.8	7.6±0.5	7.7±1.2	7.1±0.8
Male TGR + NS	4.8±1.1	6.7±0.7	39.8±3.7*	6.7±0.6	36.5±2.9*	8.1±0.9	41.5±2.7*	6.9±1.2
Male TGR + NS + B	5.3±0.9	7.4±0.5	8.8±2.8	6.4±0.6	9.1±2.9	7.7±0.6	9.3±2.4	7.4±1.3
Male TGR + HS	35.8±3.7*	7.0±0.5	75.9±3.9*+	6.7±0.7	75.1±2.7*+	6.6±0.6	75.9±3.8*+	6.8±1.1
Male TGR + HS + B	6.5±1.3	7.3±0.6	31.5±3.3*	6.4±0.8	37.2±4.1*	6.4±0.7	36.4±3.9*	7.7±1.1
Female HanSD + NS	4.6±1.2	7.4±0.8	4.1±0.6	7.1±0.9	4.2±0.9	7.2±0.4	4.7±1.2	6.8±0.9
Female HanSD + NS+ B	4.8±1.1	7.0±0.7	5.9±0.9	7.2±0.6	4.4±0.7	7.1±0.7	4.9±1.1	7.3±0.7
Female HanSD + HS	5.4±1.3	7.1± 0.9	5.1±0.7	7.2±0.5	5.7±0.9	7.2±0.5	5.8±1.2	7.1±0.6
Female HanSD + HS + B	5.2±0.9	7.2±0.8	4.7±0.9	7.3±0.6	5.1±0.8	7.0±0.5	5.3±1.1	6.9±0.8
Female TGR + NS	5.1± 0.8	6.9±0.8	5.1±1.3	7.0±0.8	4.5±0.7	7.2±0.6	4.4±0.7	7.2±0.6
Female TGR + NS+ B	4.7±0.9	7.1±0.6	4.6±1.2	7.4±0.9	4.3±0.8	7.3±0.7	4.7±0.8	7.2±0.5
Female TGR + HS	5.4±1.1	7.3±0.7	37.5±3.4*	6.1±0.3	20.8±2.3*	7.1±0.5	19.5±2.1*	6.8±0.6
Female TGR + HS + B	5.3±1.2	7.2±0.8	5.3±1.1	6.3±0.7	5.9±1.1	7.2±0.6	6.3±1.4	7.1±0.5

HanSD, transgene-negative rats; TGR, heterozygous transgenic rats (mRen2)27; NS, normal salt diet; HS, high salt diet; B – bosentan, C_{Cr}, clearance of endogenous creatinine (expressed per gram of body weight). * P<0.05 vs. HanSD rats; + P<0.05 untreated TGR fed HS vs. TGR fed NS or HS and treated with bosentan. Data shown in this table at each time point are from animals that survived until the end of the experiment (180 days of age).

Proteinuria, clearance of endogenous creatinine and urinary electrolyte excretion

All male and female HanSD fed either the NS or the HS diet exhibited minimal proteinuria and stable renal function (measured as clearance of endogenous creatinine) throughout the entire experimental period. Bosentan treatment did not affect these parameters in any of the experimental groups (Table 2)

As shown in Table 2, heterozygous male TGR fed the NS diet exhibited a progressive increase in proteinuria throughout the experimental period reaching final values that were eight times higher than those in male HanSD rats ($p<0.05$). Proteinuria was significantly reduced in rats treated with bosentan ($p<0.05$). The HS diet caused a significant increase in proteinuria in untreated heterozygous male TGR compared with NS fed animals ($p<0.05$). Bosentan treatment again significantly reduced the proteinuria in heterozygous male TGR fed the HS diet. In contrast to males, heterozygous female TGR fed the NS did not show any significant increase in proteinuria throughout the entire experiment compared with female HanSD rats. HS diet increased proteinuria in heterozygous female TGR compared with NS fed littermates ($p<0.05$), but the onset of this change was delayed and also the degree of proteinuria was lower compared with male TGR ($p<0.05$) (Table 2). Bosentan administration normalized proteinuria in heterozygous female TGR fed the HS diet to levels observed in HanSD animals.

Data on proteinuria and endogenous creatinine clearance obtained from all experimental groups throughout the study are shown in Table 2. As expected, animals fed the HS diet exhibited significantly higher daily urinary sodium excretion compared with rats fed the NS diet (between 1780 to 1980 vs. 920 to 980 µmol/24 h, $p<0.05$).

ET-1 tissue concentrations

As shown in Figure 4A, kidney cortex ET-1 concentrations in male HanSD rats were not significantly different from those in heterozygous male TGR fed the NS diet (0.20±0.04 vs. 0.24±0.05). The HS diet did not significantly change kidney cortex ET-1 levels in HanSD, but elicited a significant increase in TGR (0.23±0.03 vs. 0.48±0.04, $p<0.05$). Likewise, left ventricular ET-1 concentrations were not significantly different between HanSD and TGR rats fed the NS diet (0.42±0.06 vs. 0.56±0.09), but the HS diet caused a significant increase in TGR animals (0.49±0.05 vs. 0.78±0.06, $p<0.05$) (Fig. 4B).

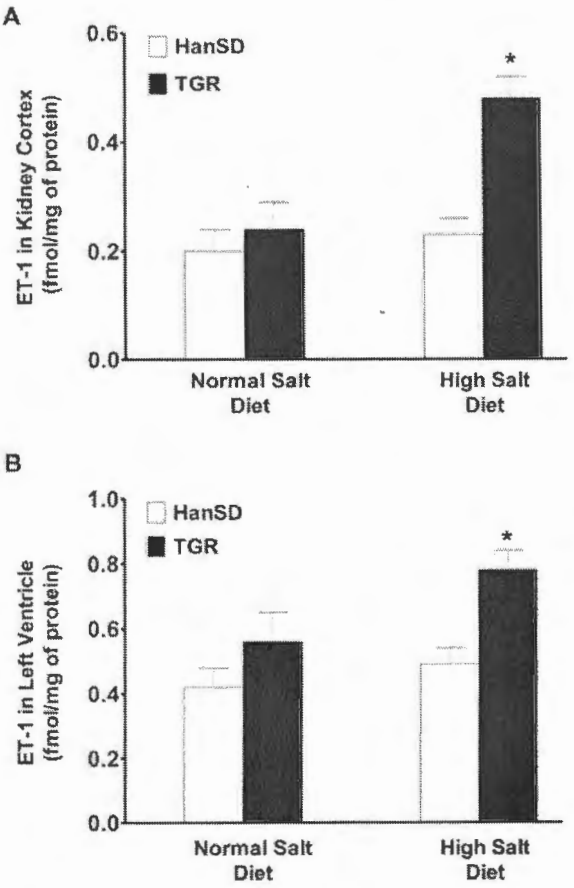

Fig. 4. Kidney cortex (**A**) and left ventricle (**B**) ET-1 levels in transgene-negative (HanSD) and homozygous male Ren-2 transgenic rats (TGR). * $P<0.05$ vs. all other values.

Discussion

The first aim of the present study was to evaluate the effects of nonselective ET_A/ET_B receptor blockade on the course of hypertension in heterozygous TGR. We found that treatment with bosentan did not alter the course of hypertension either in heterozygous male or female TGR implying a minor ET-dependent component in this model of hypertension. Our findings confirm the results obtained by Rossi´s group (Rossi *et al.* 2000, Seccia *et al.* 2003), but do not support previous findings that suggest a major contribution of the ET system to the maintenance of hypertension in TGR (Gardiner *et al.* 1995, 2000, Kelly *et al.* 2000). We cannot offer a fully satisfactory explanation for this discrepancy, but one possibility should be considered. In the aforementioned studies TGR of various ages were used, i.e. young rats were used in studies with no effect of bosentan, whereas old ones were employed in studies reporting a hypotensive effect of ET blockade. In addition, it has

been recently reported that the ET_A receptor blockade attenuated the development of hypertension in adult Dahl salt-sensitive rats but not in young animals (Dobešová *et al.* 2003). Therefore, it is pertinent to assume that ET system contributes to the pathogenesis of hypertension in mature but not in immature TGR. However, the newest study of Rothermund *et al.* (2003) does not support this concept. They demonstrated that ET receptor blockade did not decrease BP in heterozygous TGR with already established hypertension. The claim that the role of ET system in the pathogenesis of hypertension in TGR is more pronounced in adult than in young animals is in agreement with the fact that pressor systems such as vasopressin or angiotensin are less important in salt-dependent hypertension of immature rats compared to that of adult rats (for review see Zicha and Kuneš 1999). It is clear that more detailed studies are needed to address this issue.

However, with respect to the lack of BP lowering effect of bosentan treatment in TGR, the nonselective nature of this compound should be considered. Emerging body of evidence indicates that hypertensinogenic and target-organ damaging ET-1 actions are produced *via* ET_A receptor activation especially in the models of malignant and salt-loaded hypertension (d´Uscio *et al.* 1997, Orth *et al.* 1998, Rothermund *et al.* 2001, for review see Moreau and Schiffrin 2003). In addition, Vassileva *et al.* (2003) have recently demonstrated that activation of ET_B receptors participates in the pressure-natriuresis mechanism and it has been proposed that ET may participate *via* this mechanism in the long-term regulation of BP. Therefore, it is logical to assume that the selective ET_A blockade would have a BP lowering effect in TGR. However, the recent results from Rossi´s and Rothermund´s groups do not support this notion. They found that neither bosentan nor a selective ET_A receptor antagonist prevented the development of hypertension or did not lower BP in TGR (Seccia *et al.* 2003, Rothermund *et al.* 2003). Moreover, the expectation that ET_A receptor activation leads to vasoconstriction and ET_B receptor stimulation causes vasodilatation may be oversimplified, because it is known that two distinct subtypes of ET_B receptors exist (ET_{B1} and ET_{B2}). ET_{B1} receptors are present on the vascular endothelium and cause vasodilatation through the release of nitric oxide and prostaglandins (De Nucci *et al.* 1998), whereas ET_{B2} receptors are found on vascular smooth muscle cells and mediate non-ET_A vasoconstriction (Rasmussen *et al.* 1998). Moreover, ET_B receptors have been reported to stimulate aldosterone production (for review see Nussdorfer *et al.* 1999) and it has been recently demonstrated that aldosterone plays an important role in the development of cardiac and renal vascular fibrosis (Park and Schiffrin 2002, Pu *et al.* 2003, Seccia *et al.* 2003). Therefore, it seems that the nonselective ET_A/ET_B receptors blockade is a suitable experimental approach for the evaluation of the overall role the ET system in the development of ANG II-dependent hypertension. It is obvious, however, that more detailed studies employing selective ET receptor antagonist are needed to address the role of ET system and its specific ET receptor subtypes in the development of hypertension in TGR.

The second aim of this study was to assess the effects of HS diet on the course of hypertension and associated end-organ damage in heterozygous TGR. With respect to BP we found that HS intake accelerated the development of hypertension in heterozygous male and female TGR. In addition, our present study shows that in heterozygous TGR the HS diet caused a marked increase in mortality and deteriorated the cardiac and renal injury. Bosentan administration elicited a considerable improvement of survival rate and a substantial attenuation of end-organ damage in heterozygous male and female TGR fed the HS diet without influencing BP levels. These results are in good agreement with our recent observation made in homozygous TGR showing that nonselective ET receptor blockade resulted in a considerable improvement of survival rate and a substantial attenuation of end-organ damage in homozygous TGR fed the HS diet without altering BP levels (Dvořák *et al.* 2002a). Taken together, our data indicate that the ET-dependent component plays an important role in the development of end-organ damage in heterozygous male as well as female TGR fed HS diet. Our findings further support the notion that the interaction of ET-1 and ANG II contributes to the development of hypertension-induced end-organ damage (d´Uscio *et al.* 1997, Breu *et al.* 2000, Müller *et al.* 2000, Ficai *et al.* 2001, Müller *et al.* 2002, for review see Moreau and Schiffrin 2003).

In addition, our results suggest that heterozygous male as well as female TGR carry a salt-sensitive component of hypertension. These observations are in contrast with a report by Chung *et al.* (1993) who found no BP changes in TGR when given 8 % NaCl diet for 10 days. However, this difference might be explained by their use of adult animals (3 to 5 months old), while we

used young TGR. This explanation agrees with our findings that acceleration of hypertension occurred only between 35 to 40 days of age, whereas we did not observe any effects of HS diet on BP in elderly animals. Moreover, it is also important to mention that we used only 2 % NaCl and the differences in the salt-loading protocol might therefore account for different BP responses. Nevertheless, a previous study of Callahan *et al.* (1996) which showed that 2 % NaCl diet exacerbated the progression of hypertension in heterozygous male TGR, supports our findings.

The third aim of the present study was to evaluate if gender differences influence the course of hypertension and end-organ damage in TGR and if they would modify responses to dietary manipulation and pharmacological treatment. We found that heterozygous male and female TGR fed the HS diet exhibited similar pattern of the course of hypertension, survival rate and associated hypertension-induced end-organ damage. In addition, the response to bosentan treatment has a lot in common. However, the onset of the malignant phase of hypertension (characterized by enhanced mortality rate, increased proteinuria and body weight loss) was significantly delayed in heterozygous female TGR fed the HS diet compared with males. This is in good agreement with previous observations in various hypertensive models showing that males have higher BP and more serious hypertension-induced end-organ damage than age-matched females (for review see Reckelhoff 2001). Even if the mechanisms responsible for the gender differences in the severity of hypertension and end-organ damage are not yet fully understood, several reports suggest that the interaction between the RAS and androgen receptors accounts for these gender differences (for review see Reckelhoff 2001). This notion is further supported by recent findings that androgen receptor blockade significantly attenuated the development of hypertension and, even more important, completely prevented end-organ damage in male TGR (Baltatu *et al.* 2002).

Of special interest is our observation that BP in heterozygous female TGR fed the NS diet reached its peak between 65 to 80 days of age, then started to decrease spontaneously at the age of 130 days. At the end of the experiment it reached values which were significantly lower than those in heterozygous male TGR. Such a marked sexual dimorphism of BP changes in TGR was already reported by Cargnelli *et al.* (1998) who found that BP in heterozygous female TGR spontaneously returned to normotensive levels by the age of 250 days. It remains unclear whether the diminished expression of the Ren-2 gene throughout the lifetime in females is responsible for this BP decline, but the available data on the Ren-2 gene expression in females would rather argue against this possibility (for review see Langheinrich *et al.* 1996 and Lee *et al.* 1996). Therefore, the reasons for this sexual dimorphism of BP phenotype in heterozygous TGR are not known and will require more comprehensive studies.

The critical issue of the present study is related to the problem which are the underlying mechanism(s) responsible for the beneficial effects of bosentan treatment on survival rate and end-organ damage in heterozygous TGR fed the HS diet.

Multiple independent evidence indicate that the beneficial effects of ET blockade may be BP independent. It has been shown that antihypertensive treatment with hydralazine, reserpine and hydrochlorothiazide which decreased BP to normotensive levels, barely delayed end-organ damage in ANG II-dependent model of hypertension, whereas treatment with bosentan had markedly beneficial effect (Müller *et al.* 2000). Furthermore, it has been demonstrated in two-kidney, one-clip Goldblatt hypertensive rats that chronic ET_A receptor blockade attenuated cardiac hypertrophy without a reduction of BP (Ehmke *et al.* 1999). Moreover, two studies reported that mice overexpressing ET-1 developed pronounced end-organ damage independently of the increase in systemic BP (Hoecher *et al.* 1997, Shindo *et al.* 2002). With respect to the underlying mechanism(s), it has been found in these rats that treatment with bosentan inhibited the activation of nuclear factor-kappa B (NF-κB) and transcription factor activator protein (AP-1) in the kidney and in the heart independently of BP (Müller *et al.* 2000). Since ET-1 *via* ET_A receptor activation stimulates mitogen-activated protein kinase (MAPK) pathways which are important regulators of cell proliferation and play an important role in the inflammatory response and consequently in the fibrosis process, the alternative mechanism for the beneficial effects of ET blockade might be the inhibition of MAPK pathways (for review see Guijarro and Egido 2001, Luft 2002).

Alternatively, the beneficial effects of ET blockade might be related to the blockade of aldosterone-mediated deleterious actions on organ structure. It has recently been shown that ET-1 plays a critical role in the development of end-organ damage in aldosterone-infused

rats exposed to HS intake and that ET blockade prevented cardiac and aortic fibrosis independently of BP changes (Park and Schiffrin 2002). In addition, it has been reported that ET-1 exerts an important permissive action on basal aldosterone secretion and that treatment with bosentan decreased basal aldosterone secretion in heterozygous TGR (Andreis *et al.* 2000). This notion is further supported by recent findings that ET receptor blockade in aldosterone-infused rats decreased oxidative stress (Pu *et al.* 2003). Moreover, ET-1 markedly stimulates tissue superoxide production (Li *et al.* 2003). As heterozygous TGR exposed to salt-loading exhibit enhanced tissue ET-1 concentrations, it is likely that they also increased tissue superoxide levels. Since it is well known that oxidative stress increases the activity of NF-κB (Alexander 1995) and that oxidative stress plays an important role in ANG II-dependent models of hypertension (for review see Reckelhoff and Romero 2003), it seems conceivable that the beneficial effects of the ET blockade may be attributed to the blockade of the deleterious effects of aldosterone and oxidative stress on organ structure. Further studies are needed to elucidate the exact underlying mechanism(s) of our experimental observation regarding the beneficial effects of bosentan treatment.

In conclusion, we demonstrated in the present study that the chronic treatment with bosentan substantially improved the survival rate in heterozygous TGR given HS diet in the absence of BP reduction. Administration of bosentan in these groups of TGR also markedly decreased cardiac hypertrophy and renal damage, thus providing considerable protection from end-organ damage. Our data suggest that the interaction of the RAS and ET system plays an important role in the development of end-organ damage in heterozygous TGR exposed to salt-loading.

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II.B Nejdůležitější závěry experimentální studie

V naší studii jsme prokázali, že neselektivní blokáda endotelinových receptorů Bosentanem neovlivňuje vývoj hypertenze u heterozygotních transgenních potkanů nesoucích myší Ren2 reninový gen (TGR(mRen2)27). Dá se tedy shrnout, že endotelinový systém nehraje významnou roli v rozvoji vysokého krevního tlaku u tohoto modelu hypertenze.

Dalším důležitým závěrem této studie bylo zjištění, že potrava s vysokým obsahem soli u heterozygotních TGR zvířat akceleruje rozvoj arteriální hypertenze. Tedy heterozygotní TGR potkani mají sůl senzitivní složku hypertenze.

Neselektivní blokáda ET_A a ET_B receptorů pomocí Bosentanu u této skupiny zvířat na vysokoslané dietě pak výrazným způsobem snižuje mortalitu a hypertenzní orgánové poškození.

Třetím výsledkem naší studie bylo zjištění, že nejsou pohlavní rozdíly u heterozygotních TGR potkanů na vliv vysokoslané diety na rozvoj hypertenze. U samičích TGR(mRen2)27 zvířat na vysokoslané dietě však bylo dokumentováno signifikantní opoždění vývoje maligní fáze hypertenze.

II.C Časná blokáda endotelinových receptorů u hypertenzních heterozygotních Ren2 transgenních potkanů

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Early-onset endothelin receptor blockade in hypertensive heterozygous Ren-2 rats

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Abstract

Male heterozygous Ren-2 transgenic rats and Hannover Sprague–Dawley rats fed a normal or high-salt diet were either untreated or treated with the nonselective receptor ET_A/ET_B receptor blocker bosentan or the selective ET_A receptor blocker, ABT-627, known as atrasentan. Survival rate was partly increased by bosentan and fully normalized by atrasentan. Bosentan did not significantly influence the course of hypertension in TGR, whereas atrasentan significantly decreased BP on both diets. Atrasentan substantially reduced proteinuria, cardiac hypertrophy, glomerulosclerosis and left ventricular ET-1 tissue concentration on both diets. Our data indicate that ET_A receptor blockade is superior to nonselective blockade in attenuating hypertension, end-organ damage and improving survival rate.

Keywords: Endothelin receptors; Bosentan; Atrasentan; Ren-2 rats; End-organ damage

1. Introduction

Almost two decades ago, the potent vasoconstrictor peptide endothelin-1 (ET-1) was discovered (Yanagisawa et al., 1988). Its action is mediated by two types of receptors, namely ET_A and ET_B receptors. While ET_A receptors in the vascular system mediate vasoconstriction and are localized on vascular smooth muscle cells, the major function of ET_B receptors, localized mainly on endothelial cells, seems to be vasodilation in addition to its clearance function. Moreover, two distinctive ET_B receptors–ET_{B1} and ET_{B2}–with quite opposing function were identified in the rat (Gellai et al., 1996).

Since its discovery, a growing body of evidence has been accumulated showing that ET-1 plays a pivotal role in several cardiovascular diseases, including chronic heart failure, ischemic heart disease, hypertension, atherosclerosis, pulmonary hypertension and chronic heart failure. In these disease states, the levels of circulating ET-1 are increased, and treatment with ET inhibitors proved to be advantageous (Masaki, 2004). Thus, e.g. the nonspecific ET receptor blocker bosentan has been approved as a therapeutic agent to treat pulmonary hypertension (Kenyon and Nappi, 2003). However, despite the increasing evidence that the ET system plays an important role in the pathogenesis of systemic arterial hypertension, its mechanism of action is still not well understood.

Our present experiments were therefore performed, first, to evaluate the role of ET-1 in the onset and maintenance of hypertension in heterozygous Ren-2 transgenic rats. Heterozygous rats transgenic for the mouse renin gene (TGR) (strain name TGR(mRen2)27), a model of monogenetically defined hypertension (Mullins et al., 1990), exhibit a salt-sensitive component (Callahan et al., 1996). Special emphasis was given to the difference in action between the nonselective ET_A/ET_B and the specific ET_A receptor blockade. The benefit of specific

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ET_A receptor blockade may relate to the fact, that, contrary to the nonspecific receptor blockade, it does not inhibit the vasodilatory (de Nucci et al., 1988) and natriuretic (Konishi et al., 2002) response to stimulation of ET_B receptors. Besides their vasoconstrictory function, ET_A receptors also mediate cell proliferation of various cell types, especially of vascular smooth muscle cells (Hirata et al., 1989). Therefore, the blockade of ET_A receptors may be beneficial in attenuating vascular alterations leading to end-organ damage. This antiproliferative action of ET_A receptor blockers has even attracted the attention of researchers in the field of cancer research (Salani et al., 2002; Nelson, 2003).

Since dietary sodium plays an important role in the pathogenesis of hypertension not only in humans (Weinberger, 1996) but also in salt-sensitive models of hypertension (Dahl et al., 1968), our second aim was to evaluate the influence of high-salt intake on the course of hypertension, end-organ damage and survival, as well as the potential role of ET-1 in TGR under these conditions. It is generally accepted that young animals are more susceptible to various hypertensinogenic stimuli (Zicha and Kunes, 1999) and also that therapeutic interventions made in these early periods of life are more effective. However, discrepant results were reported in heterozygous TGR, i.e. either no effect (Rothermund et al., 2003a) or hypotensive (Gardiner et al., 2000) effects of nonselective or selective ET receptor blockade were found in adult animals, whereas no effect was found in young animals (Whitworth et al., 1995; Rossi et al., 2000). Therefore, in the present study we evaluated the effects of nonselective as compared to selective ET receptor blockade in TGR on different sodium diets when treatment with receptor blockers was started early in their life.

2. Materials and methods

The protocols in the present study were performed in accordance with the "Guide for the Care and Use of Laboratory Animals" and were approved by Czech Animal Care and Use Committee (Protocols 79/2001 and 923/2003).

2.1. Animals

We used male heterozygous rats transgenic for the mouse renin gene [TGR; strain name TGR(mRen2)27] and male Hannover Sprague–Dawley rats (HanSD) as normotensive controls. Animals were housed under standard conditions and had free access to chow and water. All animals used in this study were bred at the Center for Experimental Medicine of the Institute for Clinical and Experimental Medicine from stock animals supplied from Max Delbrück Center for Molecular Medicine in Berlin, Germany.

2.2. Experimental protocol

Animals were fed either a normal salt (NS, 0.45% NaCl) or high-salt diet (HS, 2% NaCl) starting on day 29 of age. At this time point, either nonselective ET_A/ET_B receptor blockade by bosentan, or selective ET_A receptor blockade by atrasentan was initiated. Bosentan (Actelion, Alschwil, Switzerland) was added to the diet. The concentration in the food was calculated to deliver a dose of 100 mg kg^{-1} day^{-1} (Roux et al., 1999). This dose was previously validated in our laboratory to effectively block ET receptors (Dvorak et al., 2004). The selective ET_A receptor blocker atrasentan (Abbott, Chicago, USA) was added to the drinking fluid; the dose was adjusted weekly to provide a concentration of 5 mg kg^{-1} day^{-1} (Mulder et al., 2000), which is generally accepted to effectively block ET_A receptors (Opgenorth et al., 1996; D'Angelo et al., 2005). At the start of the experiments, the animals were allotted to eight groups receiving either normal salt (NS) or high-salt diet (HS). As controls age-matched HanSD rats on the same regimens were investigated.

The following experimental groups were studied:

HanSD + NS (n = 24)
TGR + NS (n = 18)
TGR + NS + bosentan (n = 18)
TGR + NS + atrasentan (n = 18)
HanSD + HS (n = 24)
TGR + HS (n = 24)
TGR + HS + bosentan (n = 18)
TGR + HS + atrasentan (n = 23).

2.3. Blood pressure, proteinuria and tissue weight

From day 29 onwards, regular measurements of body weight and systolic BP (SBP) were made at weekly intervals using the tail plethysmography method (Hatteras Instruments, Cary, North Carolina, USA). At the age of 50 and 80 days, animals were housed in metabolic cages so that fluid intake could be monitored and urine collected. Urinary protein concentration in 24 h urine was measured by a biuret method (Lachema, Czech Republic).

By day 90, animals were weighed, anesthetized with thiopental sodium (50 mg kg^{-1}) and mean arterial pressure (MAP) was monitored directly in the carotid artery using the data acquisition system PowerLab (ADInstruments, Mountain View, California, USA). Kidneys and hearts were weighed. Ratios of kidney weight/body weight (KW/BW) and heart weight/body weight (HW/BW) were used as indices of organ hypertrophy.

2.4. Tissue ET-1 concentrations

Left heart ventricles were rapidly removed and cortex from the right kidney was quickly dissected. Both tissues were immediately frozen in liquid nitrogen for ET-1 determination using an enzyme-linked immunosorbent assay test (ELISA) (Amersham, Braunschweig, Germany).

2.5. Histological examination

The left kidney was quickly removed, fixed in 4% buffered formaldehyde, dehydrated and embedded. Paraffin sections

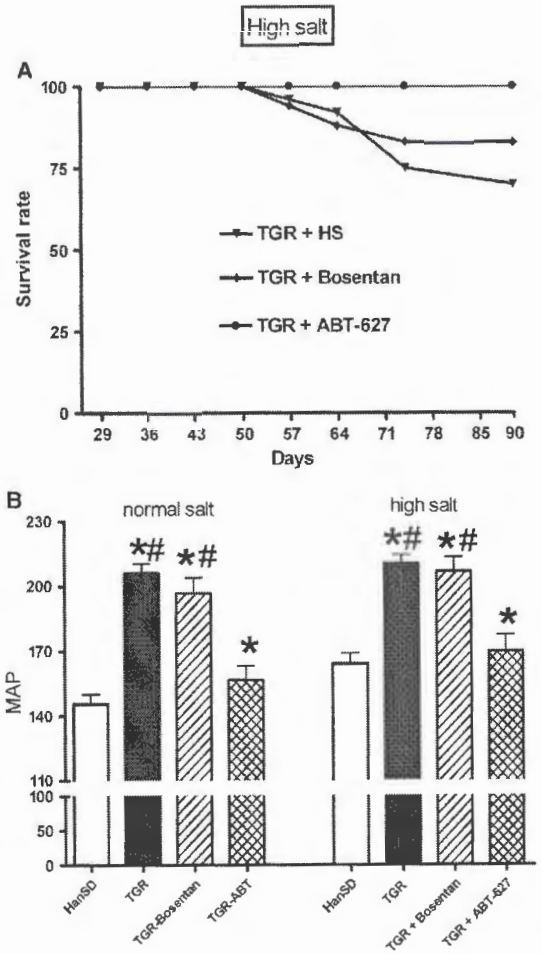

Fig. 1. Survival rate during the course of the experiment on high-salt diet (A) and mean arterial pressure on termination of the experiment (day 90) (B) in heterozygous male Ren-2 transgenic rats (TGR; +/−) on normal or high-salt intake. $^{*}p<0.05$ versus unmarked values, $^{\#}p<0.05$ versus all other values.

were stained with hematoxylin eosin and periodic acid-Schiff reaction and examined using a Nicon Eclipse E 600 light microscope. Slides were evaluated in a blind fashion. As described previously (Yagil et al., 2002), fifty glomeruli were examined on a semi-quantitative scale: grade 0=all glomeruli normal; grade 1=1–2 glomeruli affected; grade 2=more than 2 but less than 17 glomeruli affected; grade 3=17 or more glomeruli affected.

2.6. Statistical analysis

Statistical analysis of data was performed using Graph-Pad Prism software (Graph Pad Software, San Diego, California, USA). The data were evaluated by two-way ANOVA with repeated measures. We have two grouping factors (diet and therapy) and one within factor (measurement). Newman–Keuls post hoc test was used for multiple comparison. Statistical comparisons of the results obtained for heart and kidney weights and for ET-1 concentrations were made by one-way ANOVA. Unless noted, values are expressed as mean±S.E.M. and n represents the number of animals. A p-value less than 0.05 was considered significant.

3. Results

3.1. Survival rate

All TGR groups on NS diet survived to the end of the experiment. Untreated and bosentan-treated TGR on the HS diet started to die on day 50 (Fig. 1A) and their survival rates on termination of the experiment were 70% and 83%, respectively. In contrast, the administration of atrasentan markedly improved survival to 100% at the end of the experiment. Zero mortality was found in HanSD rats on both diets.

3.2. Systolic and mean arterial blood pressure

SBP of untreated and bosentan-treated TGR rose constantly throughout the course of the experiment on both diets, reaching on day 78 on the NS diet 234.6±3.6 and 214.2±4.1 mm Hg,

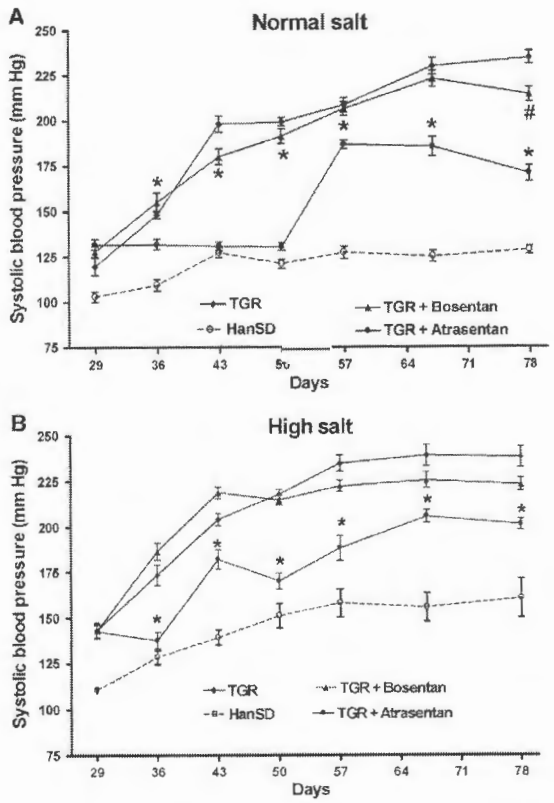

Fig. 2. Systolic blood pressure during the course of the experiment in heterozygous male Ren-2 transgenic rats (TGR; +/−) on normal (A) or high-salt (B) intake. $^{*}p<0.05$ unmarked values, $^{\#}p<0.05$ bosentan-treated versus

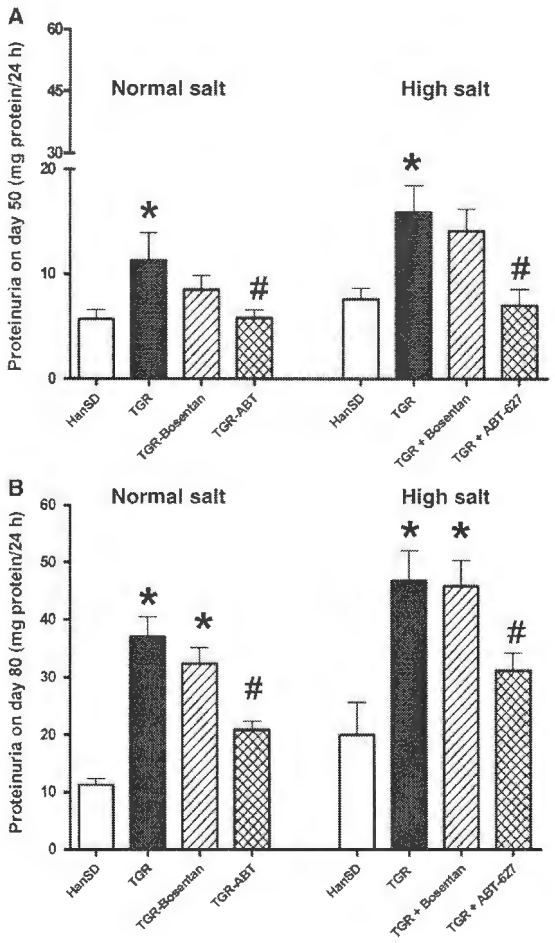

Fig. 3. Proteinuria at the age of 50 days (A) and 80 days (B) in heterozygous male Ren-2 transgenic rats (TGR; +/−) on normal or high-salt intake. *$p<0.01$ versus control HanSD, #$p<0.01$ versus untreated and/or bosentan-treated TGR.

respectively ($p<0.05$) (Fig. 2A) and on HS diet 238.2±5.8 and 223.1±3.5 mm Hg, respectively (n.s.) (Fig. 2B). From day 36 onwards (i.e. within 1 week of commencement of atrasentan treatment), SBP in atrasentan treated TGR on both diets was significantly lower than in untreated and bosentan-treated TGR, reaching on day 78 on the NS diet 170.7±4.3 mm Hg and on the HS diet 201.2±3.0 mm Hg ($p<0.01$). A highly significant increase in BP during the whole experiment induced by HS diet was found in all examined groups. SBP in control HanSD was significantly lower than in corresponding TGR groups on both diets and remained in the normotensive range during the whole experimental period, with 128.6±2.4 on the NS diet and 140.6±10.7 mm Hg on the HS diet. At the end of the experiment, MAP was significantly higher in untreated and bosentan-treated TGR than in their nonmotensive controls either on the NS (206.0±4.4 and 196.6±7.2 mm Hg, respectively; n.s.) or on the HS diet (210.2±3.9 and 206.6 ±6.7; n.s.) (Fig. 1B). On the contrary, atrasentan treatment resulted in a substantially lower MAP (156.6±6.5 on the NS and 169.9±7.4 mm Hg on the HS, $p<0.01$) when compared with untreated and bosentan-treated animals.

3.3. Proteinuria

HanSD on the NS diet exhibited low proteinuria on day 50 (5.6±0.92 mg protein/day) (Fig. 3A), which was only moderately increased by the HS diet (7.59±1.05 mg protein/day), and, as expected, both values had further increased on day 80 (11.56±1.15 on NS and 19.96± 5.78 mg protein/day on HS, respectively) (Fig. 3B). In contrast, protein excretion of untreated TGR on NS and HS diets was significantly increased on days 50 and 80, being more prominent on day 80. With bosentan treatment, there was a tendency for a partial reduction of proteinuria, while atrasentan treatment normalized protein excretion almost to the levels observed in HanSD animals.

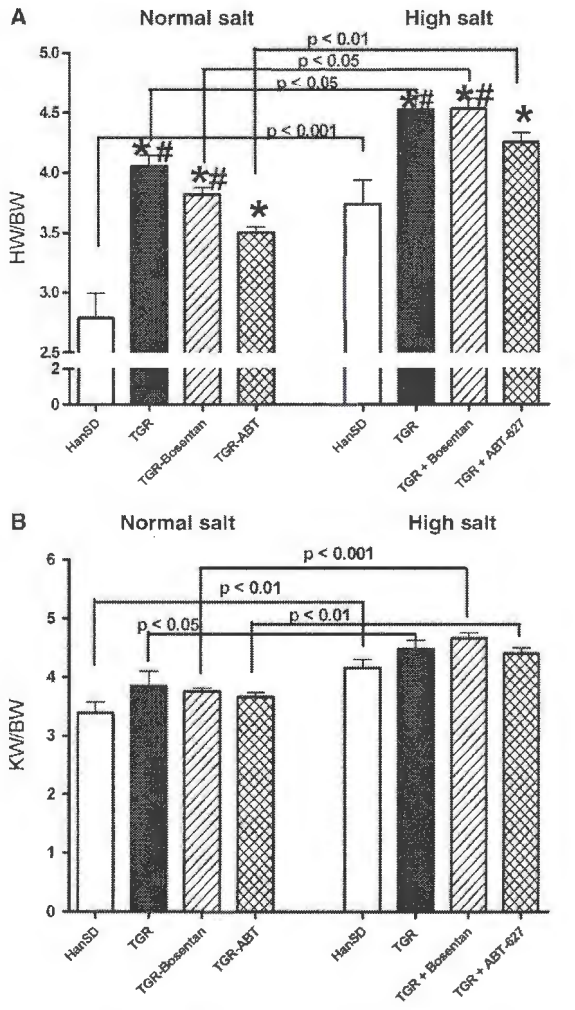

Fig. 4. Indices of HW/BW (A) and KW/BW (B) in heterozygous male Ren-2 transgenic rats (TGR; +/−) on normal or high-salt intake. Indices given in arbitrary units. *$p<0.05$ versus unmarked values, #$p<0.05$ versus all other values.

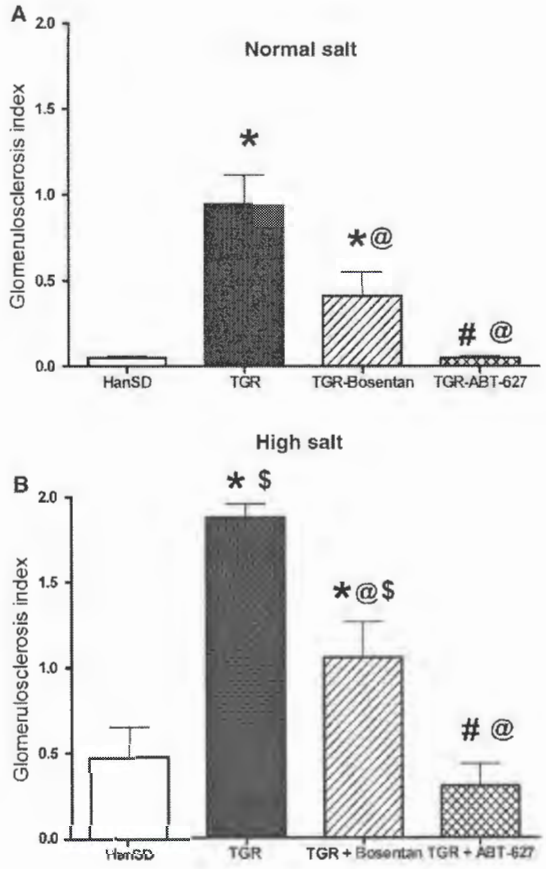

Fig. 5. Glomerulosclerosis indices in heterozygous male Ren-2 transgenic rats (TGR; +/−) on normal (A) or high-salt (B) intake. *$p<0.05$ versus control HanSD, #$p<0.05$ versus bosentan-treated TGR, @$p<0.05$ versus untreated TGR, $ versus corresponding group on NS diet.

3.4. Body and organ weights

No significant differences in body weight gains were observed between TGR groups throughout the course of the experiment; moreover, the dietary regimens had no influence on body weight gain (data not shown). Indices of HW/BW and KW/BW are depicted in Fig. 4. As expected, untreated TGR showed a significantly higher index of HW/BW than HanSD (4.06±0.09 versus 2.79±0.21, respectively, on NS and 4.53±0.12 versus 3.74±0.20, respectively, on HS diet) (Fig. 4A). Treatment with bosentan had no effect on HW/BW ratio (3.82±0.06 on NS and 4.53±0.09 on HS). On the contrary, administration of atrasentan caused a substantial decrease of HW/BW ratio both on NS (3.54±0.05) and on HS diet (4.26±0.08). HS diet induced greater cardiac hypertrophy than NS diet in all groups of rats.

No significant differences in the KW/BW indices among all examined groups were observed either on NS or HS diets; however, indices of KW/BW were significantly increased in rats on the HS as compared to rats on the NS diet (Fig. 4B).

3.5. Glomerulosclerosis index

There were almost no signs of glomerular injury in control HanSD rats on both diets (Fig. 5A and B). However, a substantial increase in glomerulosclerosis was found in untreated TGR on the NS diet and that increase was further intensified by HS intake. Bosentan treatment partly decreased, whereas atrasentan fully attenuated these organ changes.

3.6. ET-1 tissue concentration

As shown in Fig. 6A, HS diet significantly increased left ventricular ET-1 content in all groups of animals. Left ventricle ET-1 content of untreated TGR substantially exceeded that of HanSD. Both, bosentan and especially atrasentan, significantly reduced ET-1 concentrations in heart ventricles even below HanSD. Similarly, ET-1 in the kidney cortex (Fig. 6B) was

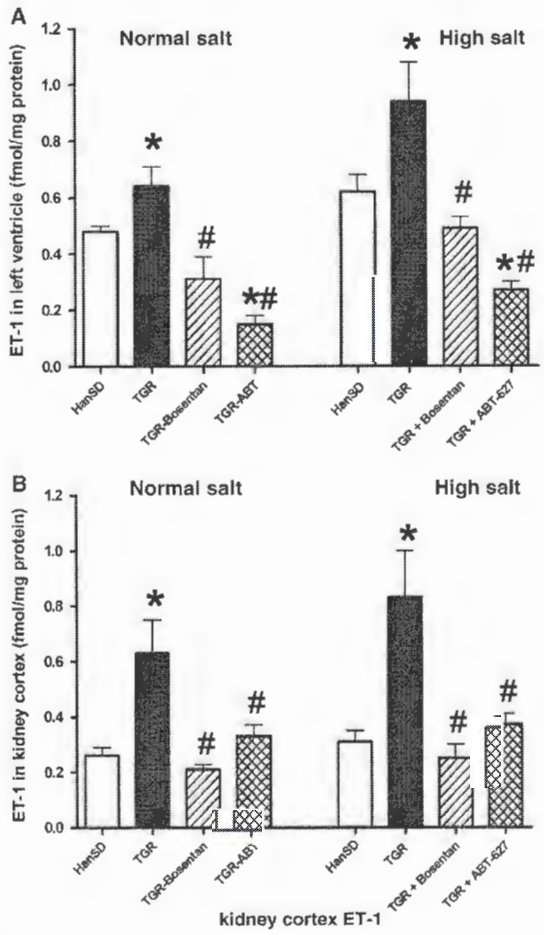

Fig. 6. Endothelin-1 concentrations in the left ventricle (A) and in the kidney cortex (B) in HanSD rats (−/−) and in heterozygous male Ren-2 transgenic rats (TGR; +/−) on normal or high-salt intake without and with bosentan or atrasentan treatment. *$p<0.05$ versus control HanSD, #$p<0.05$ versus untreated TGR.

significantly higher in untreated TGR than in HanSD. Interestingly, a more pronounced decrease in kidney ET-1 concentration was found in bosentan-treated than in atrasentan-treated animals. No significant difference was observed between rats on the HS and rats on the NS intake.

4. Discussion

Heterozygous TGR provide a suitable model of hypertension, since–in contrast to homozygous TGR who develop severe malignant hypertension–their hypertension is milder, thus allowing long-term studies. Accordingly, the *first goal* of our current study was to compare the potentially beneficial effects of a nonselective ET_A/ET_B with a selective ET_A receptor blockade in young heterozygous TGR on a *normal salt intake*. Since our previous study (Opocensky et al., 2004) has shown that chronic treatment of heterozygous TGR on a high-salt diet with the nonselective ET_A/ET_B receptor blocker bosentan substantially improved the survival rate and reduced end-organ damage without altering BP, *the second goal* of our study was to evaluate the influence of *high-salt intake* on the course of hypertension, the degree of end-organ damage, and the survival in young heterozygous TGR, as well as the potential effects of ET receptor blockade.

In our current study, in which the treatment period was half as long as in the previous one (Opocensky et al., 2004), we have first confirmed part of our previous findings, namely that the nephroprotective effects of bosentan were independent of BP changes. We then extended this observation especially to study the effects of a selective ET_A receptor blockade on hypertension and related end-organ damage. With respect to the lack of a BP lowering effect of bosentan, our findings are in accordance with previous observations in young TGR reported by Whitworth et al. (1995) and Rossi et al. (2000). In fact, increasing evidence indicates that the hypertensinogenic and organ damaging effects of ET-1 are mediated via ET_A receptor activation especially in the models of salt-dependent hypertension (Moreau and Schiffrin, 2003). On one hand, it is therefore not surprising that we found a substantial BP lowering effect of ET_A receptor blockade with atrasentan in our young heterozygous TGR. On the other hand, we have no plausible explanation that Rossi et al. (2000) did not find any decrease in BP with selective ET_A receptor blockade except that they used a different ET_A receptor blocker, namely BMS-182874, during a shorter treatment period. However, discrepant results with ETA receptor blockers were also found in other experimental hypertensive models. While no influence on BP was found in one-clip two-kidney hypertension (Ehmke et al., 1999; Saam et al., 2003) and in double renin transgenic rats (Bohlender et al., 2000), a BP lowering effect of ET_A receptor blockade was detected in the Sabra salt-sensitive strain (Rothermund et al., 2003b), in DOCA-salt rats (Allcock et al., 1998), and in stroke-prone SHR (Blezer et al., 1999). However, only in the latter experimental model studies were performed in young animals thus resembling the conditions of our present study. Moreover, our previous experiments in homozygous TGR support the idea of a substantial impact of ET_A receptor blockade on BP at an early age (Vaneckova et al., 2005), while a decreasing effect of this receptor blockade was found with increasing age (Opocensky et al., submitted for publication). The difference in the efficacy of nonselective versus selective blockade is probably due to the fact that the latter inhibits only the vasoconstrictory ET_A receptors and not the vasodilatory ET_B receptors mediating the release of NO and prostaglandins (de Nucci et al., 1988). In addition, the role of ET_B receptors as promotors of natriuresis has been shown in ET_B-deficient rats, which became hypertensive when placed on a high-salt diet (Gariepy et al., 2000). As a cause of hypertension in this model, the lack of normal functioning renal tubular epithelial sodium channels has been suggested (Gariepy et al., 2000). In these animals, selective ET_A blockade decreased systolic BP (Elmarakby et al., 2004). Interestingly, recent studies with renal cross-transplantation have shown that salt sensitivity is related probably to ET_B receptors localized extrarenally (Ohkita et al., 2005).

Regarding the second goal of our study, namely to evaluate the influence of high-salt intake on the course of hypertension, the degree of end-organ damage, and the survival in heterozygous TGR, as well as the potential effects of ET receptor blockade, we found that high-salt diet significantly accelerated the development of hypertension in heterozygous TGR. Moreover, the effect of high-salt intake on the course of hypertension was significant not only in untreated animals but also in bosentan- and atrasentan-treated TGR showing clearly that this strain carries a salt-sensitive component (Callahan et al., 1996). In addition, high-salt diet increased mortality and worsened renal and cardiac damage in this strain. Contrary to our previous finding in heterozygous TGR (Opocensky et al., 2004), in the present study bosentan was only partly effective in opposing these effects. One possible explanation of the smaller efficacy of bosentan might be that in the present study treatment with bosentan lasted only half the time of that in our previous one. In contrast, atrasentan substantially reduced both SBP and MAP, improved survival and had substantial nephro- and cardioprotective effects.

Similar to our previous study on homozygous TGR (Vaneckova et al., 2005), left ventricular ET-1 content, which was substantially lower in our heterozygous than in homozygous TGR, was decreased only partly with bosentan but to a significantly greater extent with atrasentan treatment. This correlates with the findings of Whitworth et al. (1995), who found increased preproET-1 mRNA expression in their animals with severe hypertension. Lower left ventricular ET-1 concentration following atrasentan treatment is in accordance with the findings of Barton et al. (1998) in Dahl salt-sensitive rat, who observed a decreased ET-1 protein content after ET_A receptor blockade, which is probably due to the greater displacement of ET-1 from the ET_A receptors predominating in this tissue.

In line with the recent work of Ortmann et al. (2004), who have shown in aged Wistar rats with podocyte injury a positive effect of selective ET_A receptor blockade on glomerulosclerosis and proteinuria, we also found a similar reduction of renal injury in our heterozygous TGR. Although the exact mechanism is not known, a possible explanation may be derived from the influence of ET receptor blockade on podocytes, which are the

gatekeepers of albumin passage and which are supposed to be affected by ET.

The above-mentioned findings show that the crucial question of the difference between the effectivenes of selective versus nonselective receptor blockers requires further investigations. The unselective blockade suppresses not only vasoconstrictory and proliferative actions mediated by ET_A receptors but concomitantly blocks the vasodilatory actions—mediated through the release of nitric oxide and prostaglandins (de Nucci et al., 1988)—and natriuretic actions mediated by ET_B receptors (Konishi et al., 2002). Even more confusing are the results of a very recent study of Inscho et al. (2005), which supports the idea that both types of ET receptors mediate vasoconstriction of renal afferent arterioles and that there exists a possible interaction between ET_A and ET_B receptors in controlling afferent arteriolar diameter. An interaction of ET_A and ET_B receptors has already been proposed by Just et al. (2004), who—in evaluating the physiological function of the ET system—speculated that not only dual actions of ET_B receptors but also their interaction with ET_A receptors must be taken into account.

5. Conclusions

Taken together, our results strongly support the suggestion that high-salt intake accelerates hypertension and associated end-organ damage in young heterozygous TGR when treated from weaning. They also show that, in contrast to nonselective ET receptor blockade, specific blockade of ET_A receptors exerts substantial positive effects on BP, organ damage and survival on both normal and especially on the high-salt diet. Selective ET_A receptor blockade may provide a new tool for the treatment of salt-sensitive hypertension.

Acknowledgements

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II.C Nejdůležitější závěry experimentální studie

Závěry této studie potvrdily výsledky předchozích studií, tedy pozitivní efekt blokády endotelinových receptorů neselektivním blokátorem Bosentanem na snížení mortality a hypertenzního orgánového poškození (především ledvinného parenchymu) bez ovlivnění arteriálního krevního tlaku.

Blokáda selektivním blokátorem ET_A receptorů Atrasertanem u heterozygotních TGR potkanů (TGR(mRen2)27) pak zcela normalizovala míru mortality oproti kontrolní skupině zvířat. Zároveň byla u tohoto ET_A blokátoru pozorována významná redukce proteinurie, kardiální hypertrofie, glomerulosklerózy a byly sníženy tkáňové koncentrace Endotelinu-1 v levé srdeční komoře.

Opět bylo potvrzeno, že vysokoslaná dieta u tohoto heterozygotního modelu hypertenze akceleruje rozvoj hypertenze, zhoršuje hypertenzní orgánové postižení a zvyšuje mortalitu. Stejný efekt potravy s vysokým obsahem soli byl pozorován i u obou skupin léčených neselektivním blokátorem - Bosentanem nebo selektivním blokátorem ETA receptorů - Atrasertanem. Přesto Atrasertan významným způsobem snížil systolický i střední arteriální tlak a měl nefro- a kardioprotektivní efekt.

III. Klinické studie

Endotelinové blokátory a ACE inhibitory u kardiovaskulárních onemocnění v klinických studiích

Použití ACE inhibitorů má v klinické medicíně své zcela pevné a důležité léčebné indikace. Použití blokátorů receptorů endotelinového systému je zatím testováno v několika probíhajících klinických studií.

Prozatím největšího klinického použití dosáhly endotelinové blokátory v léčbě plicní hypertenze. Je dokázáno, že hladké buňky cév a cévní epitelie produkují ET-1, zvláště pokud jsou stimulovány cytokiny. U pacientů s primární plicní hypertenzí byla popsána korelace mezi sérovými hladinami ET-1, plicní cévní rezistencí, tlakem v pravé síni a saturací kyslíku.

Bosentan (Tracleer, Actelion - USA), jako neselektivní endotelinový blokátor, se stal nejvíce testovaným lékem u plicní hypertenze. První větší studie u tohoto typu onemocnění byla provedena Channickem (Channickem R. et al, 2001). Jednalo se o randomizovanou, placebo kontrolovanou, dvojitě slepou studii s 32 pacienty. Léčba Bosentanem zlepšila primární cílový parametr studie - 6 minutovou chůzi, která se zlepšila o 70 metrů. Zlepšení bylo dosaženo i v sekundárním cílovém parametru - hodnotě srdečního indexu. Tato studie vedla k zahájení větší studie BREATH-1 (Bosentan Randomized Trial on Endothelin Antagonist Therapy) s 213 pacienty (Rubin LJ. et al., 2002). Taktéž u této studie byly zaznamenány podobné výsledky, avšak u 14% pacientů došlo k vzestupu jaterních enzymů. Přesto v listopadu 2001 schválil americký úřad pro kontrolu léčiv (Food and Drug Administration) Bosentan v dávce 2x 125 mg k léčbě plicní hypertenze.

Další studie BREATH-2 byla randomizovaná, dvojitě slepá klinická studie, která posuzovala kombinaci léčby Bosentanem spolu s epoprostenolem (prostacyklin) oproti léčbě placebo s epoprostenolem (Humbert M. et al., 2004). Tato studie však nezjistila signifikantní rozdíl mezi oběma skupinami.

Nově se nyní ve stejné indikaci zkouší selektivní blokátor ET_A Sitaxsentan oproti placebu v klinické studii STRIDE-1 (Sitaxsentan To Relieve ImpaireD Exercise) o 178 pacientech (Barst RJ. et al., 2004). Doposud jsou hlášeny pozitivní výsledky tohoto nového léku, avšak i u tohoto preparátu se při vyšším dávkování objevily elevace jaterních enzymů (21%).

Další oblast, kde antagonisté endotelinových blokátorů vstoupily do fáze klinického zkoušení, je městnavé srdeční selhání. V této indikaci byl zkoušen neselektivní blokátor TeZosentan ve studii RITZ (Ransomized Intravenous TeZosentan). Bohužel v této indikaci nebyly shledány žádné rozdíly v primárních ani sekundárních cílových parametrech studie a popsaly v několika případech stavy hypotenze. Ojediněle bylo dokumentováno i renální selhání. Studie byla několikrát opakována v různých modifikacích RITZ-2, 4, 5, avšak s velmi podobnými výsledky (Kaluski E. et al., 2003).

Stejně tak neselektivní blokátor Bosentan ve studii REACH-1 (Research on Endothelin Antagonism in Chronic Heart Failure) a ENABLE I/II (ENdothelin Antagonist Bosentan for Lowering Events) nepřinesl jednoznačné výsledky umožňující nasazení tohoto léku mezi standardní léčebné postupy.

Příznivější zprávy jsou o poslední studii HEAT (Heart Failure ET_A Receptor Blockade Trial) se selektivním blokátorem ET_A Darusentanem, která přinesla zlepšení v hlavním sledovaném parametru - srdečním indexu a nežádoucí účinky byly pozorovány pouze ve skupině pacientů s vyššími dávkami léků (Rich S. et al., 2003).

V rámci své klinické práce na I. dětské klinice a později na Pediatrické klinice FN v Motole 2. lékařské fakulty UK, jsem se zapojil do klinických studií, které řešily terapii arteriální hypertenze a hypertenzního orgánového poškození v populaci dětských pacientů. Tyto studie se týkaly úrovně kontroly hypertenze u dětí po provedené transplantaci ledviny. V léčbě těchto pacientů se jako klíčové léky dobré kontroly arteriální hypertenze zdají být inhibitory angiotenzin konvertujícího enzymu (ACEI).

III. A Kontrola hypertenze u dětí po transplantaci ledviny

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Pediatric Transplantation

Control of hypertension in children after renal transplantation

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Abstract: The aim of this cross-sectional single-center study was to investigate the efficacy of hypertension control in children who underwent transplantation using ambulatory blood pressure (BP) monitoring, and to determine the risk factors associated with poor control of hypertension. Thirty-six children fulfilled the inclusion criteria. The mean age was 13.9 ± 4.4 yr; the mean time after renal transplantation was 2.7 ± 2.4 yr (0.5–10.1). Hypertension was defined as a mean ambulatory BP ≥ 95th centile for healthy children and/or requiring antihypertensive drugs. Hypertension was regarded as controlled if the mean ambulatory BP was < 95th centile in children already on antihypertensive drugs, or uncontrolled if the mean ambulatory BP was ≥ 95th centile in treated children. Hypertension was present in 89% of children. Seventeen children (47%) had controlled hypertension, and 14 (39%) had uncontrolled hypertension. One child (3%) had untreated hypertension, and only four children (11%) showed normal BP without antihypertensive drugs. The efficacy of hypertensive control was 55% (17 of 31 children on antihypertensive drugs had a BP < 95th centile), i.e. 45% of treated children still had hypertension. Children with uncontrolled hypertension had significantly higher cyclosporine doses (6.1 vs. 4.3 mg/kg/day, $p = 0.01$) and tacrolimus levels (9.2 vs. 6.1 μg/L, $p < 0.05$), and there was a tendency toward use of lower number of antihypertensive drugs (2.0 vs. 1.5 drugs/patient, $p = 0.06$) and lower use of angiotensin-converting enzyme (ACE) inhibitors (7 vs. 35%, $p = 0.09$) and diuretics (29 vs. 59%, $p = 0.14$) than in children with controlled hypertension. In conclusion, nearly 90% of our children after renal transplantation are hypertensive and the control of hypertension is unsatisfactorily low. The control of hypertension could be improved by increasing the number of prescribed antihypertensive drugs, especially ACE inhibitors, and diuretics, or by using higher doses of currently used antihypertensives.

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Key words: arterial hypertension – renal transplantation – antihypertensive treatment – calcineurin inhibitors – left ventricular hypertrophy

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Arterial hypertension is a common complication in pediatric patients after renal transplantation (1–4). The etiology of post-transplant hypertension is multifactorial – pretransplant hypertension, damaged native kidneys, steroids, calcineurin inhibitors, and chronic allograft nephropathy being the most important causes. Hypertension is regarded as an important risk factor for cardiovascular morbidity and mortality as well as for allograft survival (5–7). Ambulatory blood pressure monitoring (ABPM) is a better method for blood pressure (BP) evaluation than casual BP measurement in children after renal transplantation (8, 9). The prevalence of hypertension in children after renal transplantation is high according to the published studies using ABPM, ranging between 36% and 76% (10–14). The control of post-transplant hypertension in clinical practice is often difficult, and the efficacy of antihypertensive therapy in transplanted children therefore seems to be low. However, no study has yet focused on the

Abbreviations: ABPM, ambulatory blood pressure monitoring; ACE inhibitors, angiotensin-converting enzyme inhibitors; BP, blood pressure; LVH, left ventricular hypertrophy; LVMI, left ventricular mass index

efficacy of antihypertensive therapy in children after renal transplantation.

The aim of our cross-sectional single-center study was to investigate prospectively the rate of control of BP and the efficacy of hypertensive therapy in children after renal transplantation, and to determine the risk factors associated with uncontrolled hypertension in our transplantation center.

Patients and methods

All 45 children after renal transplantation, followed-up in our center at the end of the year 2002, were screened for eligibility for this study. The inclusion criteria were that the children had had renal transplant at least six months before this study and had not experienced acute rejection in the last three months. During the next three months, all included children underwent measurement of BP using ABPM. At the same time echocardiography and graft function were assessed, the type of antihypertensive and immunosuppressive therapy as well as the clinical characteristics of the children were recorded.

The study was approved by the local Ethics Committee of Human Experimentation, and parental consent was obtained before the study.

Blood pressure was measured by ABPM during 24 h using a SpaceLabs 90207 (Redmond, WA, USA) oscillometric monitor; BP was automatically recorded every 20 min during daytime and every 30 min at night. The ABPM study was considered satisfactory for analysis if at least 40 recordings were obtained during the study. Patient diaries were obtained on their activities during ABPM, and the period of night-time BP (sleep period) was noted according to the diary. The mean systolic and diastolic BP at daytime and at night-time was calculated and compared with standards obtained in healthy European children (15). Hypertension was defined as a mean systolic or diastolic BP at daytime or night-time $\geq$95th centile for healthy children or use of antihypertensive drugs. Hypertension was regarded either as controlled (defined as a mean BP $<$95th centile in patients on antihypertensive drugs), or uncontrolled (defined as a mean BP $\geq$95th centile in patients on antihypertensive drugs), or as untreated (defined as a mean systolic or diastolic BP at daytime or night-time $\geq$95th centile in children without antihypertensive drugs). Blood pressure was regarded as spontaneous normotension if the mean systolic and diastolic BP values at daytime and night-time were $<$95th centile for healthy children in children without any antihypertensive drug.

Blood pressure standard deviation score (SDS) was calculated for mean systolic and diastolic BP at daytime and at night-time using the LMS method (16). Nondipping phenomenon was defined as a night-time BP decline $<$10% for systolic and/or diastolic BP.

Casual BP values (mercury sphygmomanometer) from the last three patient's visits in the renal clinic were recorded and evaluated using a standard technique (17), and the mean of these three values was assessed and compared with daytime ambulatory BP values.

A standard two-dimensional echocardiogram (GE/Wingmed, System 5, Vivid 7, Horten, Norway) was performed on the same day as ABPM according to the recommendations of the American Society of Echocardiography (18). Left ventricular mass was calculated according to the formula of Devereux from the left ventricular internal dimension at end diastole, interventricular septal thickness, and left ventricular posterior wall thickness (19). Left ventricular mass was indexed to a height$^{2.7}$ (left ventricular mass index [LVMI]) to account for body size (20). Left ventricular hypertrophy (LVH) was defined either as an LVMI $>$ 38.6 g/m$^{2.7}$, which corresponds with the 95th centile of normative pediatric LVMI data (20), or as an LVMI $>$ 51.0 g/m$^{2.7}$, which, in adults, has been found to correlate with a fourfold greater risk of cardiovascular events (21).

Graft function was estimated from the serum creatinine level (enzymatic method) using the Schwartz formula (22). All children were on steroids (daily dose in 34 children) and calcineurin inhibitors (cyclosporine or tacrolimus); all but one was on azathioprine or mycophenolate mofetil. The trough levels of calcineurin inhibitors were measured (Emit assay). Body mass index (BMI) was expressed in percentiles of the healthy Czech child population (23). Overweight was defined as a BMI $>$ 85th centile, and obesity as BMI $>$ 95th centile. Anemia was defined as a hemoglobin level $<$ 120 g/L.

Data are given as frequencies, median and range, or mean and standard deviation. Statistical analysis was performed using SPSS statistical software. Fisher's exact test or the Mann–Whitney *U*-test were used to compare the characteristics between subgroups. $p < 0.05$ was regarded as a statistical significant difference.

Results

Patients

Thirty-six of 45 children (20 boys) fulfilled the inclusion criteria and were eligible for the study. Nine children were excluded from the study because of a short interval after renal transplantation ($<$6 months, n = 7), and two children because of acute rejection in the last 3 months. The mean age at the time of the study was 13.9 $\pm$ 4.4 yr (range 4.6–19.5); the mean time after renal transplantation was 2.7 $\pm$ 2.4 yr (range 0.5–10.1). Thirty-four children had a graft from a deceased donor (DD); 33 children had a first transplantation. Previous dialysis therapy was hemodialysis in 19 patients and peritoneal dialysis in 16 patients; one child underwent pre-emptive transplantation. The clinical features and immunosuppressive therapy are summarized in Table 1.

Thirty-one children (86%) were already on antihypertensive drugs. The mean number of antihypertensives in treated patients was 2.1 $\pm$ 1.1 drugs/patient; eight children were on monotherapy and 23 were on combined antihypertensive therapy (two drugs in 15 children, three drugs in five children, four drugs in three children). Twenty-three children were on β-blockers (atenolol, betaxolol), 21 children on calcium-channel blockers (nifedipine slow release, amlodipine, isradipine), 14 children on diuretics (hydrochlorothiazide, furosemide), and seven children on angiotensin-converting enzyme inhibitors (ACE inhibitors; enalapril, ramipril). No child received calcium-

channel blockers or ACE inhibitors solely for renoprotection or proteinuria without having hypertension.

The median dosage of antihypertensive drugs (in mg/kg/day) was 0.8 for atenolol, 0.3 for betaxolol, 0.7 for nifedipine, 0.2 for amlodipine, 0.2 for isradipine, 0.4 for hydrochlorothiazide, 0.9 for furosemide, 0.2 for enalapril, and 0.03 for ramipril.

Blood pressure

Hypertension was diagnosed in 89% of children. Seventeen children (47%) had controlled hypertension and 14 (39%) had uncontrolled hypertension. One child (3%) revealed untreated hypertension and four children showed normal BP without antihypertensive drugs (11%). Hypertension (untreated or uncontrolled) was either combined daytime and night-time (60% of hypertensive children) or isolated night-time (40%). No child had isolated daytime hypertension. Hypertension was either combined systolic and diastolic (60% of hypertensive children) or isolated systolic (40%). No child had isolated diastolic hypertension.

The efficacy of antihypertensive therapy defined as the control of hypertension was 55% (17 of 31 children on antihypertensive drugs were normotensive), i.e. 45% of treated children were still hypertensive. The comparison of clinical features and immunosuppressive and antihypertensive therapy between children with controlled and uncontrolled hypertension is given in Table 2.

The mean BP SDS in transplanted children is summarized in Table 1. All BP SDS with the exception of daytime diastolic BP were statistically significantly higher than BP SDS in healthy children ($p<0.01$ for daytime systolic BP and $p<0.001$ for night-time systolic and night-time diastolic BP, (16)). No significant correlation was found between systolic or diastolic daytime or night-time BP (expressed in SDS) and estimated creatinine clearance.

The comparison of clinical features and immunosuppressive therapy between children with hypertension ($n = 32$) and spontaneous normotension ($n = 4$) revealed a significant difference in the frequency of nephrectomy of native kidneys (16 vs. 75%, $p = 0.02$) and in the frequency of using alternate dose of prednisone (0 vs. 50%, $p = 0.01$). All other tested variables (as in Table 1) showed no significant differences (data not shown).

The prevalence of nondipping phenomenon was 64%. The mean systolic BP dip was $7.2 \pm 4.7\%$, and the mean diastolic BP dip was $12.9 \pm 5.6\%$. Both systolic and diastolic BP dip in transplanted children were statistically significantly reduced compared with $13\% \pm 6\%$ and $23\% \pm 9\%$ in the normal pediatric population (15). Three children (8%) had inverted circadian BP rhythm (i.e. night-time mean BP higher than daytime mean BP). No significant correlation was found between systolic or diastolic BP dip and estimated creatinine clearance.

Table 1. Clinical characteristics, immunosuppressive therapy, and ambulatory blood pressure in 36 children after renal transplantation

	Frequency (number of patients in parentheses) or median (range in parentheses)
Hypertension prior transplantation	42% (n = 15)
Nephrectomy of native kidneys (uni- or bilateral)	22% (n = 8)
Congenital primary renal disease*	58% (n = 21)
Calculated creatinine clearance (mL/min/1.73 m^2)	73 (38–127)
Acute rejection (one or more)	39% (n = 14)
Chronic rejection (biopsy proven)	6% (n = 2)
Graft artery stenosis corrected previously by balloon angioplasty	6% (n = 2)
Body mass index (centile)	53 (1–99)
Overweight	25% (n = 9)
Obesity	14% (n = 5)
Prednisone dose (mg/kg/day)	0.11 (0.04–0.31)
Cyclosporine (% of all children)	50% (n = 18)
Cyclosporine dose (mg/kg/day)	4.9 (2.8–7.6)
Cyclosporine level (μg/L)	141 (36–224)
Tacrolimus (% of all children)	50% (n = 18)
Tacrolimus dose (mg/kg/day)	0.12 (0.05–0.22)
Tacrolimus level (μ/L)	7.4 (2.4–12.0)
Daytime systolic blood pressure (SDS, mean ± SD)	+0.60 ± 1.46
Daytime diastolic blood pressure (SDS, mean ± SD)	−0.09 ± 1.80
Night-time systolic blood pressure (SDS, mean ± SD)	+1.57 ± 1.33
Night-time diastolic blood pressure (SDS, mean ± SD)	+1.10 ± 1.51

*Congenital primary renal diseases include congenital hypoplasia, dysplasia, reflux nephropathy, obstructive uropathy, nephronophthisis, cystinosis, congenital nephrotic syndrome of the Finnish type.

The mean casual systolic BP did not differ from the mean daytime systolic BP (125 mmHg [103–153] vs. 122 mmHg [92–145], $p = 0.06$) but the mean casual diastolic BP was significantly higher than the mean daytime diastolic BP (80 mmHg [61–100] vs. 71 [49–94], $p = 0.0003$).

Echocardiography

Left ventricular hypertrophy was present in 50% of children using the 95th centile of normative pediatric LVMI data for definition and 14% using the adult definition. The mean LVMI in all children was 41.0 ± 9.8 g/$m^{2.7}$. There was no significant difference in LVMI between normotensive

Table 2. Clinical characteristics and immusuppressive and antihypertensive therapy in hypertensive children after renal transplantation treated with antihypertensive drugs (controlled and uncontrolled hypertension)

	Controlled hypertension (n = 17)	Uncontrolled hypertension (n = 14)	p value
Hypertension prior transplantation	59% (n = 10)	36% (n = 5)	NS*
Nephrectomy of native kidneys (uni- or bilateral)	18% (n = 3)	14% (n = 2)	NS*
Congenital primary renal disease	47% (n = 8)	79% (n = 11)	NS*
Calculated creatinine clearance (mL/min/1.73 m^2)	77 (38–127)	69 (46–108)	NS†
Acute rejection (one or more)	29% (n = 5)	43% (n = 6)	NS*
Chronic rejection (biopsy proven)	6% (n = 1)	7% (n = 1)	NS*
Graft artery stenosis corrected previously by angioplasty	0% (n = 0)	14% (n = 2)	NS*
Body mass index (centile)	70 (1–99)	45 (3–99)	NS†
Overweight	29% (n = 5)	29% (n = 4)	NS*
Obesity	12% (n = 2)	21% (n = 3)	NS*
Prednisone dose (mg/kg/day)	0.11 (0.05–0.31)	0.11 (0.06–0.23)	NS*
Cyclosporine (% of all children)	41% (n = 7)	57% (n = 8)	NS*
Cyclosporine dose (mg/kg/day)	4.3 (3.4–4.9)	6.1 (2.8–8.0)	0.01†
Cyclosporine level (μg/mL)	146 (36–224)	108 (47–161)	NS†
Tacrolimus (% of all children)	59% (n = 10)	43% (n = 6)	NS*
Tacrolimus dose (mg/kg/day)	0.12 (0.04–0.22)	0.11 (0.05–0.19)	NS†
Tacrolimus level (μg/L)	6.1 (2.4–8.9)	9.2 (4.6–12.0)	0.045
Number of antihypertensive drugs per patient	2.0 (1–4)	1.5 (1–4)	NS† (0.06)
Calcium channel blockers (% of children)	65% (n = 11)	71% (n = 10)	NS*
β-blockers (% of children)	77% (n = 13)	71% (n = 10)	NS*
Diuretics (% of children)	59% (n = 10)	29% (n = 4)	NS* (0.14)
ACE inhibitors (% of children)	35% (n = 6)	7% (n = 1)	NS* (0.09)

The data are percentages or medians with range (in parentheses).
*Fisher's exact test,
†Mann–Whitney *U*-test.
ACE inhibitors indicates angiotensin-converting-enzyme inhibitors.
NS, not significant difference.

and hypertensive children (41.4 vs. 40.3 g/$m^{2.7}$), between children with controlled and uncontrolled hypertension (38.5 vs. 39.3 g/$m^{2.7}$), between children with normal and abnormal nocturnal BP dip (43.4 vs. 39.8 g/$m^{2.7}$), or between children with anemia and without anemia (36.4 vs. 40.2 g/$m^{2.7}$). There was no significant correlation between LVMI and daytime or night-time systolic or diastolic BP values expressed in SDS. No correlation was found between LVMI and estimated creatinine clearance or hemoglobin level.

Discussion

In our cross-sectional study, we could demonstrate that 89% of children after renal transplantation were hypertensive and that the efficacy of antihypertensive treatment was unsatisfactorily low – only 55% of hypertensive children had their hypertension well controlled by antihypertensive drugs, and one child had untreated hypertension. The prevalence of persistent hypertension despite antihypertensive treatment (i.e. prevalence of uncontrolled hypertension) was rather high also in several recent pediatric studies that focused primarily on the prevalence of hypertension. Hypertension ranged between 47% and 82% in these studies using ABPM (8, 10–12, 14). This means that only 18% to 53% of children after renal transplantation had well-controlled hypertension.

The reasons for the low efficacy of antihypertensive therapy in transplanted patients have not been well investigated (24). Many factors, such as chronic allograft nephropathy, require lifelong use of BP-elevating immunosuppressive drugs (steroids, cyclosporine, tacrolimus), obesity, salt retention, renin secretion from diseased native kidneys, undertreatment with low doses of antihypertensive drugs, and the fear of ACE inhibitors in transplanted patients (risk of acute renal failure in patients with renal artery stenosis of the graft) are discussed as the major reasons for inadequate BP control in patients who undergo transplantation (4). Lastly, noncompliance can play an important role, particularly in adolescent patients. We could demonstrate in our study that the only risk factors for uncontrolled hypertension were significantly higher cyclosporine doses and tacrolimus levels and a tendency toward use of lower number of antihypertensive drugs and lower use of ACE inhibitors and diuretics. Calcineurin inhibitors have known hypertensive effects, and the mechanisms of hypertensinogenic effects of cyclosporine and tacrolimus are believed to be

multifactorial. They include increase in systemic vascular resistance, vasoconstriction of the glomerular afferent arteriole, increase in sympathetic nervous tone, activation of the renin–angiotensin system, impaired nitric oxide-dependent vasodilatation, or sodium and water retention (4, 25, 26). Gordjani et al. (27) showed that high trough levels of cyclosporine (>400 ng/mL) were associated with a significantly higher incidence of hypertension in comparison with children with levels <400 ng/mL (91 vs. 57%). Furthermore, hypertensive children had slightly higher cyclosporine levels than normotensives but the difference was not significant.

The trend in undertreatment by antihypertensive drugs in patients with uncontrolled hypertension (lower number of antihypertensives) supports the hypothesis that post-transplant hypertension is not truly resistant to the therapy but that it is not appropriately treated. The daily doses of prescribed antihypertensive drugs in our cohort were in the recommended ranges (17), although mostly in the lower part of the range. However, children with controlled hypertension often received two times more diuretics and five times more ACE inhibitors than children with uncontrolled hypertension. Although these differences did not reach statistical significance, we think that they are of clinical relevance. The results of a recent retrospective study on ACE inhibitors in children further underline this concept (28). The authors could achieve adequate control of hypertension in 100% of transplanted children one yr after addition of an ACE inhibitor in patients with refractory hypertension. Whether the use of ACE inhibitors can improve the BP control also in a prospective study still needs to be investigated. The current results on the control of post-transplant hypertension highlight a high potential for improvement of the antihypertensive therapy in children after renal transplantation. Control of hypertension could be improved by increasing the number of prescribed antihypertensive drugs, especially ACE inhibitors and diuretics, or by using higher doses of the current most commonly used classes of calcium-channel blockers and β-blockers. Another potential tool could be reduction of the dose of steroids or calcineurin inhibitors that are not only hypertensinogenic but also nephrotoxic. Better BP control could improve the long-term graft as well as patient survival.

Risk factors for hypertension

In our study, only the presence of both native kidneys (i.e. patients without nephrectomy of their native kidneys) and the use of a daily dose of steroids were significant risk factors for development of hypertension. These findings are in agreement with previous data showing that the presence of native kidneys, hypertension prior to transplantation, and steroid dose are important risk factors for persistence or new development of post-transplant hypertension (27, 29, 30). Several other authors reported that nephrectomy of the native kidneys before renal transplantation protects against the persistence of hypertension after renal transplantation, or that BP decreases after native kidney nephrectomy in transplanted patients (27, 30, 31).

The role of steroids in post-transplant hypertension is well known (29, 32). Several factors such as sodium retention or increase in cardiac output and renal vascular resistance induce steroid-related hypertension. Elimination of steroids in stable patients showed reduction of BP in adult as well as in pediatric patients (33, 34), which is in good agreement with our study where the patients on alternate-day steroid treatment showed a significantly lower prevalence of hypertension than children on daily-steroid medication. We did not find any differences in cyclosporine or tacrolimus dose or level between children with hypertension and normotension. Similarly, in the only randomized-controlled trial comparing cyclosporine and tacrolimus-based immunosuppression in pediatric patients who underwent renal transplantation, there were no significant differences in the prevalence of hypertension between children treated with cyclosporine and those treated with tacrolimus (35). However, other studies in adults showed less hypertension in patients on tacrolimus than on cyclosporine (36), and therefore this issue still remains unresolved.

ABPM evaluation

An interesting finding of our study as well as of the studies performed by Giordano et al. (12) and Morgan et al. (14) is the lack of isolated daytime hypertension in these patients. This finding indicates that if a child has hypertension after renal transplantation, it is always night-time hypertension, either isolated night-time or combined with daytime hypertension, but never isolated daytime hypertension. This finding further underlines the importance of ABPM with its monitoring of BP values during night that are always elevated if the child is hypertensive.

Left ventricular hypertrophy

We could demonstrate that LVH is a frequent finding in transplanted children. The prevalence of

50% is similar to that in other pediatric studies using the same pediatric definition (11, 14, 37). Furthermore, the mean LVMI in our study (41 $g/m^{2.7}$) was the same as in the most recent report on cardiac function in children who underwent transplantation (38). We could not find any difference in LVMI between normotensive and hypertensive children and any correlation between LVMI and ambulatory BP values. This is in contrast to the study performed by Matteucci et al. (11), who found a correlation between LVMI and mean 24 h systolic BP, but is in agreement with more recent cross-sectional studies performed by Morgan et al. (14) and Kitzmueller et al. (39), who also could not find any correlation between LVMI and ambulatory BP data. Similarly, Mitsnefes et al. (38) did not find any relationship between LVMI and BP, although they measured only clinic BP. However, Kitzmueller et al. (39) found a correlation between LVMI and ABPM data on repeated measurement, suggesting that control of BP is important for the maintenance of the myocardial architecture.

In conclusion, we demonstrated that 89% of children are hypertensive after renal transplantation, that 40% of them have isolated night-time hypertension detectable by ABPM only, and that the control of hypertension is low – only 55% of treated children have normal ambulatory BP. The dose and blood level of calcineurin inhibitors, together with a lower number of antihypertensive drugs, namely ACE inhibitors and diuretics, were the risk factors for poor BP control in hypertensive children. Control of hypertension could be improved by increasing the number of prescribed antihypertensive drugs, especially ACE inhibitors.

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III. A Nejdůležitější závěry této klinické studie:

V této průřezové studii bylo zjištěno, že 89% dětí po provedené transplantaci ledviny má arteriální hypertenzi a že účinnost antihypertenzní léčby u těchto pacientů je pouze 55%. Jako hlavní rizikové faktory nedostatečné kontroly hypertenze po transplantaci ledviny u dětí byly detekovány dávka a hladiny kalcineurinových inhibitorů spolu s malým počtem podávaných antihypertenziv. Především se jedná o nedostatečně předepisované ACE inhibitory a diuretika.

Z hypertenzního orgánového postižení byla zjištěna hypertrofie levé komory srdeční u poloviny transplantovaných dětí.

Tato průřezová studie se stala východiskem pro naší následující – intervenční studii, která měla za cíl zlepšit kontrolu nedostatečně léčené hypertenze u transplantovaných dětí a snížit výskyt hypertenzního orgánového postižení.

III. B Zlepšení kontroly arteriální hypertenze u dětí po transplantaci ledviny: Výsledky dvouleté intervenční studie

Pediatr Transplantation 2007: 11: 491–497

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Improved control of hypertension in children after renal transplantation: Results of a two-yr interventional trial

Seeman T, Šimková E, Kreisinger J, Vondrák K, Dušek J, Gilík J, Dvořák P, Janda J. Improved control of hypertension in children after renal transplantation: Results of a two-yr interventional trial. Pediatr Transplantation 2007: 11: 491–497. © 2007 Blackwell Munksgaard

Abstract: Hypertension is a frequent complication in children after renal transplantation and the control of post-transplant hypertension is unsatisfactorily low. The aim of this prospective interventional study was to improve the control of hypertension in children after renal transplantation. Thirty-six children fulfilled the inclusion criteria (≥6 months after transplantation and no acute rejection in the last three months). BP was measured using ABPM. Hypertension was defined as mean ambulatory BP ≥95th-centile for healthy children and/or using antihypertensive drugs. The study intervention consisted of using intensified antihypertensive drug therapy – in children with uncontrolled hypertension (i.e., mean ambulatory BP was ≥95th centile in treated children), antihypertensive therapy was intensified by adding new antihypertensive drugs to reach goal BP < 95th centile. ABPM was repeated after 12 and 24 months. Daytime BP did not change significantly after 12 or 24 months. Night-time BP decreased from 1.57 ± 1.33 to 0.88 ± 0.84 SDS for systolic and from 1.10 ± 1.51 to 0.35 ± 1.18 SDS for diastolic BP after 24 months ($p < 0.05$). The number of antihypertensive drugs increased from 2.1 ± 0.9 to 2.7 ± 0.8 drugs per patient ($p < 0.05$), this was especially seen with the use of ACE-inhibitors (increase from 19% to 40% of children, $p < 0.05$). In conclusion, this interventional trial demonstrated that, in children after renal transplantation, the control of hypertension, especially at night-time, can be improved by increasing the number of antihypertensive drugs, especially ACE-inhibitors.

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Key words: arterial hypertension – renal transplantation – antihypertensive treatment – angiotensin-converting enzyme inhibitors – diuretics – calcineurin inhibitors – graft function – left ventricular hypertrophy

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Hypertension is a common complication in pediatric patients after renal transplantation affecting 60–80% of children depending on the method of BP measurement and definition of hypertension (1–9). It is a well-known risk factor for impaired allograft survival as well as for patient survival in both adult and pediatric patients after renal transplantation (10–13). The control of post-transplant hypertension in clinical practice is inadequately low. In several studies using ABPM, the best method for BP evaluation in transplanted children, only 18–53% of children demonstrated well-controlled hypertension (5–7, 9, 14–15). In our cross-sectional study, we could confirm these findings (only 55% of our children had controlled hypertension – i.e., 45% of treated children still had hypertension). However, we have shown that an important risk factor for poor control of hypertension was undertreatment, i.e., a low number of antihypertensive drugs being used, especially ACE-inhibitors and diuretics (16). Post-transplant hypertension seems to be more undertreated rather than resistant to the therapy. Therefore, the aim of our interventional study

Abbreviations: ABPM, ambulatory blood pressure monitoring; ACE-inhibitors, angiotensin converting enzyme inhibitors; BP, blood pressure; DD, deceased donor; LVH, left ventricular hypertrophy; LVM, left ventricular mass; LVMI, left ventricular mass index; SDS, standard deviation score.

was to improve the poor control of hypertension in transplanted children by a more rigorous antihypertensive treatment plan.

Patients and methods

All 36 children after renal transplantation who fulfilled inclusion criteria for our previous cross-sectional study were included in this two-yr interventional study. The inclusion criteria were: children that have had a renal transplantation at least six months prior to this study and that had not experienced acute rejection in the last three months. All included children underwent measurement of BP using ABPM. Echocardiography and graft function were assessed at the same time, the type of antihypertensive and immunosuppressive therapy together with the clinical characteristics of the children were recorded.

Blood pressure was measured by ABPM during 24-h using a SpaceLabs 90207 (Redmond, WA, USA) oscillometric monitor: BP was automatically recorded every 20 min during daytime and every 30 min at night. The ABPM study was considered satisfactory for analysis if a minimum of 40 recordings were obtained during the study. Patient diaries were obtained, illustrating their activities during ABPM and the period of night-time BP (sleep period) was noted according to the diary. Mean systolic and diastolic BP at daytime and at night-time was calculated and compared with standards obtained in healthy European children (17). Hypertension was defined as mean systolic or diastolic BP at daytime or night-time ≥95th centile for healthy children or use of antihypertensive drugs. Hypertension was regarded either as controlled (defined as a mean BP < 95th centile in patients on antihypertensive drugs), or uncontrolled (defined as a mean BP ≥95th centile in patients on antihypertensive drugs) or as untreated (defined as a mean systolic or diastolic BP at daytime or night-time ≥95th-centile in children not using antihypertensive drugs). BP was regarded as spontaneous normotension if mean systolic and diastolic BP values at daytime and night-time were < 95th centile for healthy children in children without any antihypertensive drug.

Blood pressure SDS was calculated for mean systolic and diastolic BP at daytime and at night-time using the LMS-method (18). Non-dipping phenomenon was defined as night-time BP decline < 10% for systolic and/or diastolic BP. Clinic BP values (mercury sphygmomanometer) obtained from the patient's last three visits to the renal clinic were recorded and compared with standards for clinic BP in healthy children using the 95th centile for the definition of hypertension (19). The mean of these three values was assessed and compared with daytime ambulatory BP values. Clinic BP index was calculated by dividing subject BP value by the 95th centile BP value.

A standard two-dimensional echocardiogram (GE/Vingmed system 5; Vivid 7, Horten, Norway) was performed on the same day as ABPM according to the recommendations of the American Society of Echocardiography (20). LVM was calculated according to the formula of Devereux from the left ventricular internal dimension at end diastole, interventricular septal thickness and left ventricular posterior wall thickness (21). LVM was indexed to height$^{2.7}$ (LVMI) to account for body size (20). Left ventricular hypertrophy was defined as LVMI > 38.6 g/m$^{2.7}$, which corresponds with the 95th centile of normative pediatric LVMI data (22, 23).

Graft function was estimated from the serum creatinine level (enzymatic method) using the Schwartz formula (24). Proteinuria was measured in 24-h collecting urine and expressed in mg/m^2/24-h. All children underwent Doppler ultrasound to exclude renal artery stenosis at the beginning of the trial (graft artery stenosis was corrected previously by balloon angioplasty in two children). All children were on steroid treatment (daily dose used in 34 children) and calcineurin-inhibitors (cyclosporine or tacrolimus); all but one were on azathioprine or mycophenolate mofetil. The trough levels of calcineurin-inhibitors were measured (Emit-assay, Abbott, Abbott Park, IL, USA). The targeted trough levels for cyclosporine were 100–150 μg/L in combination with azathioprine and 80–120 μg/L with mycophenolate, the targeted trough levels for tacrolimus were 5–7 μg/L.

Current study intervention

The study intervention consisted of intensifying the antihypertensive drug therapy in children with uncontrolled or untreated hypertension by ABPM. Additional antihypertensive agents were added at the discretion of the treating physician according to the following general guidelines: In children who were not receiving ACE-inhibitors or diuretics at baseline, the first agents added were ACE-inhibitors (enalapril or ramipril) or diuretics (hydrochlorothiazide or furosemide). ACE-inhibitors were the drugs of first choice in children with proteinuria or left ventricular hypertrophy. In children already receiving ACE-inhibitors, the drugs of first choice were diuretics. To better control patient adherence to the prescribed antihypertensive medication, parents or children were asked at every outpatient visit on the use of all medicaments and their dosages.

Immunosuppressive therapy was guided according to the local policy (monitoring through levels, conversion from cyclosporine to tacrolimus in case of acute rejection or serious adverse effects such as severe gingival hyperplasia requiring gingivectomy). This treatment has not been tailored because of present BP, i.e., changes of immunosuppression were not permitted because of detected hypertension.

Blood pressure was measured as clinic BP on every outpatient visit (monthly) and as ABPM after one and two yr. The goal BP was clinic systolic and diastolic BP < 95th centile and daytime and night-time systolic and diastolic BP < 95th centile for ABPM. Echocardiogram was repeated after one and two yr. The study was approved by the local Ethics Committee of Human Experimentation and consent of the parents was obtained before the study. Data are given as frequencies, median and range or mean and standard deviation. Statistical analysis was performed using SPSS statistical software. McNemar test or Wilcoxon signed rank test were used to compare the characteristics at the beginning of the study and after one and two yr and Mann–Whitney *U*-test was used to compare the characteristics between normotensive and hypertensive subgroups. $p < 0.05$ was regarded as a statistical significant difference.

Results

Patients

The mean age of children was 13.9 ± 4.4 yr (range: 4.6–19.5), mean time after transplantation was 2.7 ± 2.4 yr (range: 0.5–10.1). Thirty-four children had a graft from a DD; 33 children had had their first transplantation. Previous dialysis therapy was hemodialysis in 19 patients

and peritoneal dialysis in 16 patients; one child underwent pre-emptive transplantation. Thirty-three children completed the study after one yr and 31 children after two yr. The reasons of drop-out were graft failure because of steroid-resistant acute rejection (n = 1), transition to adult transplantation center (n = 2), non-compliance with recommended study protocol (n = 1) and psychiatric disorder (n = 1). Five children experienced acute rejection (between months 2–8 of the study), four of them were steroid sensitive.

Blood pressure, antihypertensive therapy and echocardiography

The mean number of successful BP readings per ABPM study was 58 ± 24, 57 ± 23 and 60 ± 26 readings at the start of the study, after one and two yr. Mean ambulatory BP expressed in SDS decreased after one and two yr, but the BP decrease reached statistical significance only after two yr for night-time systolic and night-time diastolic BP (Fig. 1). Night-time BP dip improved after two yr significantly. The prevalence of uncontrolled and untreated hypertension decreased but the decrease did not reach statistical significance. The mean number of prescribed ACE-inhibitors and diuretics increased significantly after two yr but the number of prescribed beta-blockers and calcium channel blockers did not change (Fig. 2). The median dosage of antihypertensive drugs (in mg/kg/day if not given otherwise) at baseline, after one and two yr was 0.2, 0.2 and 0.2 for enalapril, 1.2, 1.8 and 1.7 mg/m^2/day for ramipril, 0.9, 0.7 and 0.6 for furosemide and 0.4, 0.3 and 0.2 for hydrochlorothiazide. The changes were not statistically significant. Left ventricular mass index and the prevalence of left ventricular hypertrophy did not change significantly. No correlation was found between LVMI and ambulatory BP data after one or two yr. The data on BP, antihypertensive therapy and echocardiography during this two yr interventional trial are summarized in Table 1. The number of children on 1, 2, 3 and 4 antihypertensive drugs during the two-yr study is given in Table 2.

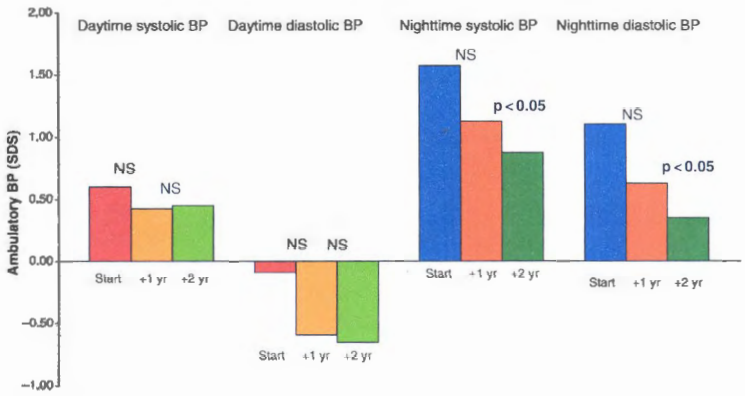

Fig. 1. Changes in ambulatory blood pressure during the study. BP, blood pressure, NS, not significant, SDS, standard deviation score.

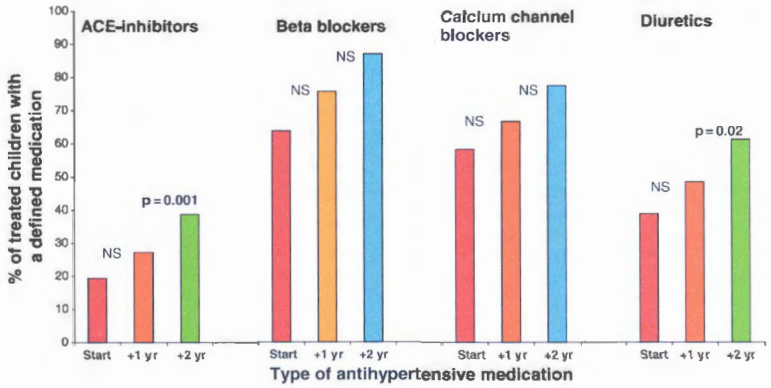

Fig. 2. Changes in antihypertensive medication during the study.

Table 1. Blood pressure and antihypertensive therapy in children after renal transplantation during two yr interventional trial

	Start of the study (n — 36)	After 1 yr (n — 33)	p-value (start vs. 1 yr)	After 2 yr (n — 31)	p-value (start vs. 2 yr)
Prevalence of uncontrolled and untreated hypertension (%)	42	36	NS	26	NS
No. antihypertensive drugs per treated pts.	2.1 ± 0.9	2.4 ± 0.77	**0.029**	2.7 ± 0.78	**0.002**
Night-time dip of systolic BP (%)	7.2 ± 4.7	8.3 ± 4.1	NS	10.2 ± 5.6	**0.036**
Night-time dip of diastolic BP (%)	12.9 ± 5.6	13.2 ± 6.9	NS	14.9 ± 7.5	NS (0.07)
Non-dippers (% of patients)	64	67	NS	45	**0.04**
Clinic systolic BP index	1.02 ± 0.11	0.97 ± 0.30	NS	0.95 ± 0.35	NS
Clinic diastolic BP index	1.00 ± 0.11	0.92 ± 0.32	**0.011**	0.93 ± 0.35	NS (0.09)

Data are frequencies or mean ± standard deviation (s.d.). NS — not significant. Significant results are shown in bold.

Table 2. The number of children on 0, 1, 2, 3 and 4 antihypertensive drugs during two-yr interventional trial

	Start of the study (n — 36)	After 1 yr (n — 33)	After 2 yr (n — 31)
No antihypertensive drugs (%)	5 (14)	2 (6)	1 (3)
1 antihypertensive drug (%)	8 (22)	2 (6)	1 (3)
2 antihypertensive drugs (%)	15 (42)	17 (52)	11 (35)
3 antihypertensive drugs (%)	5 (14)	8 (24)	13 (42)
4 antihypertensive drugs (%)	3 (8)	4 (12)	5 (16)

Renal function, proteinuria and immunosuppression

Mean calculated creatinine clearance decreased after the first year and did not change during the second year of the trial. In a subgroup of children who reached normal BP levels at two yr, no change of graft function was observed. On the contrary, in a subgroup of children who remained hypertensive at two yr, graft function impaired significantly after two yr (Fig. 3). Mean proteinuria did not change during the first year but improved significantly during the second year. No change in the percentage use of cyclosporine (50%, 36%, 35% of all children at baseline and after one and two yr) and tacrolimus, in prednisone dose, cyclosporine dose and tacrolimus level were observed. However, cyclosporine dose decreased significantly during the first year and tacrolimus level during the second year of the trial. Data on renal function, proteinuria and immunosuppression during this two-yr interventional trial are summarized in Table 3.

Side effects of study intervention

One child experienced acute rise of serum creatinine (>20% from baseline) one month after starting therapy with an ACE-inhibitor, this was fully reversible after discontinuation of the drug. No graft artery stenosis could be detected in this child, however, he had biopsy proven chronic allograft nephropathy. Another child experienced mild hypokalemia after initiation of thiazide diuretic, which normalized after reduction of the dose. In children treated with ACE-inhibitors, no child developed hyperkalemia or suffered from cough.

Discussion

This is the first interventional trial on intensified, ABPM-guided, BP treatment in children after renal transplantation. Over a two-yr period, repeated use of ABPM for the diagnosis of hypertension together with a more rigorous

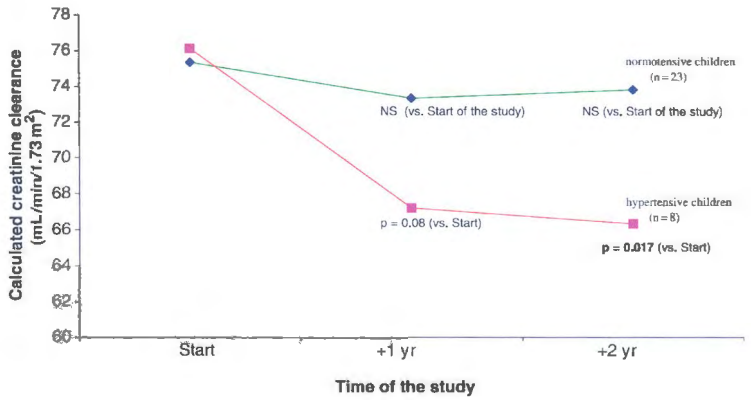

Fig. 3. Graft function in children being normotensive and hypertensive at two yr. The differences in graft function between normotensive and hypertensive children at the start of the study and after one and two yr were not statistically significant.

Table 3. Graft function, proteinuria, echocardiography and immunosuppressive therapy in children after renal transplantation during two-yr interventional trial

	Start of the study (n = 36)	After 1 yr (n = 33)	p-value (start vs. 1 yr)	After 2 yr (n = 31)	p-value (start vs. 2 yr)
Calculated creatinine clearance (mL/min/1.73 m^2)	75.5 ± 20.2	71.9 ± 19.4	**0.028**	71.9 ± 18.1	NS (0.051)
Proteinuria (mg/m^2/24 h)	256 ± 303	224 ± 208	NS	134 ± 88	**0.002**
Left ventricular mass index (g/$m^{2.7}$)	41.12 ± 9.82	39.84 ± 20.38	NS	43.71 ± 11.30*	NS
Prevalence of left ventricular hypertrophy by pediatric criteria (%)	50	43	NS	67*	NS
Prednisone dose (mg/kg/day)	0.11 ± 0.06	0.10 ± 0.06	NS	0.09 ± 0.06	NS
Cyclosporine dose (mg/kg/day)	4.9 ± 1.4	3.6 ± 1.9	**0.004**	3.4 ± 1.7	**0.003**
Cyclosporine level (μg/L)	141 ± 51	132 ± 72	NS	137 ± 64	NS
Tacrolimus dose (mg/kg/day)	0.12 ± 0.06	0.11 ± 0.07	NS	0.11 ± 0.07	NS (0.08)
Tacrolimus level (μg/L)	7.4 ± 2.6	7.0 ± 3.7	NS	5.5 ± 3.3	**0.026**

The data are frequencies or mean ± standard deviation (s.d.). NS = not significant. Significant results are shown in bold.
*Data on three patients with normal echocardiography findings at the start of the study are missing.

treatment protocol improved BP control in transplanted children. Most significantly, nocturnal BP, which has been associated with an increased risk of cardiovascular disease and LVH in adults, was improved by the study intervention. Furthermore, graft function appeared to stabilize in children who achieved and maintained normotension, but declined in those who remained hypertensive by ABPM. Previous studies on hypertension in transplanted children that show the control of hypertension is low (efficacy of antihypertensive therapy only 18–53%) have speculated on the reasons of poor control of post-transplant hypertension (3–4, 12). The most common hypothetical reason was the multifactorial etiology of post-transplant hypertension, factors such as: presence of diseased native kidneys, use of steroids, calcineurin inhibitors, suboptimal graft function, sodium retention, overweight. However, in our previous cross-sectional study, we have shown that the main risk factor for uncontrolled hypertension is a low number of antihypertensive drugs, mainly ACE-inhibitors and diuretics and that post-transplant hypertension seems not to be truly resistant to the therapy but rather that it is only not appropriately treated (16). In the current interventional trial, we have confirmed our hypothesis because we could significantly decrease BP in transplanted children and improve control of hypertension by increasing the number of antihypertensive drugs, especially ACE-inhibitors and diuretics. A similar conclusion has been drawn from a retrospective study performed by Arbeiter et al. (25) who showed that the use of ACE-inhibitors could improve BP control in children with presumed "resistant" hypertension.

All but one patient tolerated additional antihypertensive drugs very well. One boy with biopsy proven chronic allograft nephropathy exhibited acute drop of graft function after initiation of ACE-inhibitors, which was fully reversible after discontinuation of the drug. Several retrospective studies have demonstrated an association between BP and long-term allograft survival in adult and pediatric patients (2–3, 10–11, 13). Nevertheless, no prospective interventional trials showing that lowering BP can improve graft survival have been published so far. However, a recent retrospective study by Opelz et al. (26) demonstrated that improved BP control in the last seven yr was associated with improved long-term graft and patient survival. Further studies provided clear evidence that not hypertension "*per se*" (defined only on the basis of the use antihypertensive drugs) but the actual BP level is the decisive factor influencing graft survival (27, 28). Transplanted patients with controlled hypertension had the same graft survival as patients with spontaneous normotension. This highlighted the paramount importance of the control of hypertension in the prevention of chronic allograft dysfunction.

In our study, graft function decreased by 3.6 mL/min/1.73 m^2 during the first year but subsequently stabilized during the second year. The stabilization of graft function during second year was associated with a significant decrease of night-time systolic as well as diastolic BP. Furthermore, children who remained hypertensive after two yr lost significant graft function in comparison with children who reached normal BP after two yr (Fig. 3). This is further evidence that elevated BP is deleterious to graft function and is not only a marker of graft dysfunction. It is consistent with the findings of Mitsnefes et al. (3) who have shown that hypertension may act as a deleterious factor for the graft function in children with normal or only slightly decreased graft function but not in children with severely impaired graft function. These data support the hypothesis that hypertension is not only a

consequence of graft dysfunction but can directly impair graft function.

Calcineurin inhibitors such as cyclosporine and tacrolimus have hypertensinogenic and nephrotoxic effects. We cannot exclude that a part of the improvement of BP control and stabilization of graft function could be due to significant declines in cyclosporine dose and tacrolimus levels that were made during routine care of transplanted children and not because of study protocol. However, a significant decrease in tacrolimus level was observed only during the second year of the study but no significant change in tacrolimus daily dose per bodyweight was noted.

Left ventricular hypertrophy is a frequent finding in transplanted children (6, 9, 29–30). We were unable to decrease the prevalence of LVH despite improvement of BP control and significant decrease of ambulatory BP. The reason for the lack of improvement of left ventricular architecture can be due to the fact that BP is not the only determinant of left ventricular architecture and that also non-hemodynamic factors such as sympathetic overactivity, renin angiotensin system, salt intake, body weight or anemia play an important role in the genesis of LVH (31). Furthermore, we could not find any correlation between LVMI and ambulatory BP values at the beginning of the study (16), which correlates with a study completed by Morgan et al. (9) but is in contrast to the study done by Matteucci et al. (6) who found a correlation between LVMI and mean 24-h systolic BP or by Kitzmueller et al. who found a correlation between LVMI and ABPM data on repeated measurement suggesting that control of BP is important for the maintenance of the myocardial architecture (32). Another reason why no change of LVMI was observed in our study could be the fact that to high treatment BP goal (95th centile) was chosen. Lower BP goal (90th centile) is recommended for children with chronic kidney diseases, however, it is not known if this lower treatment goal should be used also in children with transplanted kidneys.

Proteinuria in adults is an important risk factor not only for the progression of chronic native kidney diseases but also for poor long-term allograft as well as patient survival (33). Therefore, proteinuria together with hypertension is believed to be an important treatable risk factor for chronic allograft dysfunction. We have demonstrated in a previous cross-sectional study that proteinuria is also a frequent finding in pediatric renal transplant recipients (34). In this interventional trial, we could significantly decrease the amount of proteinuria after two yr. We can only speculate the main reason for this antiproteinuric effect. It could be due to the increased use of ACE-inhibitors, decreased BP level or stabilization of graft function during the second year of the study. Nevertheless, regardless of the reason, this positive "side effect" of our interventional trial, which focused primarily on BP control should have a positive effect on the long-term graft function because a decrease in proteinuria during treatment is associated with slower progression of chronic kidney diseases (35, 36).

In conclusion, we demonstrated in this first prospective interventional trial that BP control can be improved in children after renal transplantation by rigorous antihypertensive therapy. An increased number of prescribed antihypertensive drugs, especially ACE-inhibitors and diuretics, resulted in a decrease of BP especially during night-time. Improved BP control could also result in a decrease in proteinuria and stabilization of graft function. In the long-term, these positive findings could improve the graft function as well as graft and patient survival.

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III. B Nejdůležitější závěry klinické studie:

Tato naše studie byla první intervenční studií zabývající se léčbou hypertenze u dětí s transplantovanou ledvinou.

Ve dvouleté intervenční studii bylo prokázáno, že zvýšením počtu podávaných antihypertenzních léků, především pak ACE inhibitorů a diuretik, lze signifikantně snížit arteriální krevní tlak u dětí po provedené transplantaci ledviny.

Dále bylo prokázáno, že dobrá kontrola krevního tlaku u těchto pacientů signifikantně snižuje rychlost poklesu glomerulární filtrace během dvouletého sledování. Funkce transplantované ledviny se dokonce stabilizovala v druhém roce sledování.

Dalším zjištěním byl pak pokles míry proteinurie na konci dvouletého období této studie. Dá se však pouze spekulovat, jestli zmírnění proteinurie je dáno zejména renoprotektivním antiproteinurickým efektem častěji používaných ACE inhibitorů nebo přímo zlepšením kontroly arteriálního krevního tlaku bez ohledu na typ užívané antihypetenzní léčby.

Ani přes zlepšenou kontrolu hypertenze se nepodařilo snížit výskyt hypertenzního orgánového postižení srdce (LVH).

IV. Shrnutí nejdůležitějších poznatků výzkumné a publikační činnosti autora

Renin-angiotenzinový a endotelinový systém mají zásadní význam pro kontrolu arteriálního krevního tlaku v organizmu. V experimentální části této práce byl použit dobře definovaný, monogenní, angiotenzin II dependentní model hypertenze – transgenní potkan nesoucí myší reninový Ren2 gen (TGR (mRen2)27).

Všechny experimentální studie autora potvrdily zásadní efekt zvýšeného přísunu sodíku v dietě na rozvoj arteriální hypertenze a zhoršení hypertenzního orgánové postižení (především ledvin a srdce) u tohoto modelu hypertenze

Vzhledem k existenci více typů receptorů endotelinoveho systému – ET_A a ET_B receptory (s dvěma subtypu ET_{B1} a ET_{B2}) naše práce přinesly nové poznatky o fyziologickém a patofyziologickém fungování těchto receptorů. Neselektivní blokáda ET_A a ET_B receptorů pomocí Bosentanu významným způsobem zlepšuje přežívání homozygotních i heterozygotních TGR zvířat, snižuje míru hypertenzního orgánového poškození srdce, snižuje proteinurii a glomerulosklerózu. Tyto pozitivní účinky neselektivní endotelinové blokády nejsou způsobeny poklesem arteriální krevního tlaku. Důvod pro tuto skutečnost je dán rozdílnými účinky jednotlivých subtypů endotelinových receptorů a jejich nerovnoměrnou distribucí v organizmu. Aktivací ET_A receptorů je navozena vazokonstrikce, ET_{B1} působí přes uvolnění NO a prostaglandinů vazodilatačně a ET_{B2} receptory lokalizované na hladkých svalových buňkách cév způsobují non-ET_A mediovanou vazokonstrikci. Výsledek neselektivní blokády je pak dán souhrou všech těchto mechanismů.

Vazokonstrikční účinek aktivace ET_A receptoru byl potvrzen v poslední zmiňované experimentální práci (II.C). V této studii byl jasně demonstrován pozitivní efekt selektivní blokády ETA receptoru Atrasertanem – u experimentálních zvířat byl signifikantně snížen arteriální krevní tlak, byla snížena mortalita, došlo k redukci proteinurie, kardiální hypertrofie a glomerulosklerózy. Tyto účinky pak ve srovnání s neselektivní blokádou byly významnější. Selektivní receptorová blokáda ET_A může tedy do budoucna přinést nový léčebný nástroj na ovlivnění sůl senzitivní arteriální hypertenze.

V klinické části této disertační práce bylo prokázáno, že arteriální hypertenze je velmi častou komplikací u dětí po transplantaci ledviny, která je navíc spojena s vysokým výskytem hypertenzního poškození cílových orgánů, zejména hypertrofií levé komory

srdeční. Naše studie ukázala, že u dětí po transplantaci ledviny jsou v nedostatečné míře používány ACE – inhibitory. Jedná se o jeden z rizikových faktorů neuspokojivé kontroly krevního tlaku u těchto dětí.

Naše dvouletá intervenční studie ukázala, že hypertenze u dětí po transplantaci ledviny není rezistentní na léčbu, jak spekulovaly některé předchozí studie, ale naopak, že lze zvýšeným užíváním antihypertenzních léků, zejména ACE inhibitorů zlepšit kontrolu hypertenze.

Zlepšená kontrola hypertenze byla navíc spojena se stabilizací funkce transplantované ledviny. Bohužel ani zlepšená kontrola hypertenze nevedla v naší intervenční studii na rozdíl od zlepšení funkce transplantované ledviny k poklesu prevalence hypertenzního orgánového postižení srdce ve smyslu hypertrofie levé komory.

V. Závěr

Léčba arteriální hypertenze a s ní spojeného hypertenzního orgánového poškození je zásadní pro ovlivnění celkové morbidity a mortality kardiovaskulárních onemocnění. Přes obrovský nárůst vědomostí v oblasti endotelinového systému se zatím nepodařilo zavádět tyto znalosti ve větší míře do klinické praxe. Výsledky experimentálních studií však ukazují, že farmakologické ovlivnění endotelinového systému přináší nové, slibné možnosti léčby kardiovaskulárních onemocnění. Přispívají k tomu i nové modely hypertenze u transgenních potkanů, které jsme použili i v našich experimentech. Spektrum terapeutických indikací se bude pravděpodobně dále rozšiřovat i mimo oblast kardiovaskulárních nemocí. Důkazem pro tento názor je i rozsáhlý seznam farmakologických společností vyvíjejících nové druhy endotelinových blokátorů. Dá se spekulovat, že tyto nové léčebné možnosti budeme moci použít i v klinické praxi a to i v tak úzce specifické oblasti jako je arteriální hypertenze u dětí po transplantaci ledviny.

VI. Poděkování

Na prvním místě bych chtěl poděkovat především panu profesorovi MUDr. Janu Jandovi, CSc., za to že mne přivedl ke studiu a práci v oblasti dětského lékařství. Pod jeho vedením jsem pracoval na I.dětské klinice FN Motol a následně jsem se začal věnovat dětské nefrologii. Jako můj druhý školitel významnou měrou přispěl ke vzniku a dokončení této disertační práce.

Na druhém místě, bohužel již jen „in memoriam", patří dík panu profesorovi MUDr. Jiřímu Hellerovi, CSc., který stál na počátku mého postgraduálního studia a pomohl mi se zahájením práce v oblasti experimentální medicíny.

Zcela největší míra díků pak patří bezpochyby panu profesorovi MUDr. Luďku Červenkovi, Ph.D., který jako můj hlavní školitel, krok po kroku směroval moji experimentální práci a zcela zásadním způsobem přispěl ke vzniku zde uvedených publikací. Vzhledem k tomu, že jsem rozděloval svoji práci mezi klinickou a experimentální medicínu, měl se mnou vždy trpělivost a vždy mi dokázal se vznikem této práce všestranně pomoci. Za to bych mu chtěl vyslovit ještě jednou díky!

Rád bych poděkoval i všem pracovníkům Pracoviště experimentální medicíny (PEM), se kterými jsme na vzniku těchto experimentálních studií spolupracoval. Rád bych zde zmínil pana Horáčka, který mne naučil pracovat s experimentálními zvířaty, a vždy dokázal svojí radou pomoci při řešení problémů v laboratoři.

Rád bych poděkoval Doc. MUDr. Tomáši Seemanovi, CSc., který mi vždy jako můj konzultant při mé práci na Pediatrické klinice FN Motol dokázal poradit a směrovat moji klinickou i experimentální práci. Především díky jeho zásluze vznikly zde uvedené klinické studie z oblasti klinické hypertenze u dětských transplantovaných pacientů.

V neposlední řadě patří veliké díky mé rodině, mým rodičům a mé manželce, kteří mne vždy v mém studiu a práci maximálně podporovali. O to více mne mrzí, že se již o výsledky své práce nepodělím se svým otcem.

Ještě jednou děkuji

Pavel Dvořák

VII. Seznam publikací autora použitých k problematice disertační práce

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