UNIVERZITA KARLOVA V PRAZE
FARMACEUTICKÁ FAKULTA V HRADCI KRÁLOVÉ
KATEDRA FARMAKOLOGIE A TOXIKOLOGIE

ADHEZNÍ MOLEKULY A JEJICH ÚLOHA V MODELOVÝCH PATOLOGICKÝCH STAVECH

Disertační práce

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

Naďa Pospíšilová

OBSAH

Seznam zkratek

apoE	apolipoprotein E
apoE$^{-/-}$/LDLr$^{-/-}$	apoE/LDL – receptor deficitní - dvojnásobně knokautovaná myš
BCR	antigenně specifický receptor B lymfocytů
CD 105	endoglin
CETP	cholesteryl ester transfer protein
CRP	C – reaktivní protein
EDGF	endothelium derived growth factor
E3L	ApoE*3-Leiden transgenní myš
eNOS	endoteliální NO syntáza
Hb	hemoglobin
HDL	high density lipoprotein, lipoproteiny s vysokou hustotou
HHT	hereditární hemoragická telaengiektázie
HMG-CoA	3-hydroxyl-3-methylglutaryl koenzym A
HOCl	kyselina chlorná
ICAM-1	intercellular cell adhesion molecule, adhezivní molekula
IDL	intermediate density lipoproteins, lipoproteiny se střední hustotou
ICHS	ischemická choroba srdeční
IL-1, IL-6, IL-8	interleukin-1, -6, -8
INF-γ	interferon gamma
LDL	low density lipoprotein, lipoproteiny s nízkou hustotou
MAdCAM-1	mucosal addressin cell adhesive molecule
MCP-1	monocytární chemotaktický protein-1
M-CSF	macrophage colony stimulating factor, růstový hormon makrofágů
NF-κB	nukleární faktor kappa B
NO	oxid dusnatý
OxLDL	oxidované LDL částice
PAF	faktor aktivující destičky
PAI-1	inhibitor aktivátoru plazminogenu
PDGF	platelet-derived growth factor, destičkový růstový faktor

PECAM-1	platelet endothelial cell adhesion molekule, adhezivní molekula
p-Smad2/3	fosforylovaný Smad2/3
TbRI, TbRII	receptor pro TGF-β typu I a typu II
TCR	antigenně specifický receptor T lymfocytů
TGF β	transforming growth factor, transformující růstový faktor
TNF α	tumor necrosis factor, prozánětivý cytokin
t-PA	tkáňový aktivátor plazminogenu
VLA	very late antigens
VCAM-1	vascular cell adhesion molecule, adhezivní molekula
VLDL	very low density lipoprotein, lipoproteiny s velmi nízkou hustotou

I. ÚVOD A CÍLE PRÁCE

Ateroskleróza byla kdysi považována za mechanický proces prostého ukládání tuku do cévní stěny. Počátkem devadesátých let začala být ateroskleróza chápána jako chronický proces, na kterém se podílejí prozánětlivé molekuly, cytokiny a růstové faktory. Ateroskleróza je dnes někdy označována jako „nemoc 20. století“, je jednou z hlavních příčin kardiovaskulárních onemocnění (Daubresse 2000).

Aterosklerotický proces probíhá v několika stádiích, které v případě dlouhotrvajícího působení aterogenních faktorů mohou vést až ke vzniku klinických komplikací. Začíná adhezí oxidovaných částic LDL k cévní stěně a končí - většinou asi až o několik desetiletí později - rupturou fibrózní „čepičky“ aterosklerotického plátu, vytvořením trombu a akutní cévní příhodou, infarktem myokardu, nebo ischemickou chorobou dolních končetin. Tyto orgánové komplikace jsou nejčastější příčinou předčasné invalidizace a úmrtí ve většině civilizovaných zemí a jsou jedním z významných sociálních problémů civilizace, spojených se „západním stylem života“. Díky tomuto stylu života bude mít tento zdravotní problém mnohem širší následky zejména během následných desetiletí (Keaney 2000).

Česká republika se nachází, i přes příznivý vývoj v posledních letech, na jednom z předních míst v Evropě v úmrtnosti populace na choroby srdce a cév. Velkým problémem je stárnutí populace a s tím související zvyšování prevalence kardiovaskulárních onemocnění v populaci.

V průběhu posledního desetiletí byl zaznamenán jednoznačný posun v etiologii a patogenezi vzniku aterosklerózy, stejně jako v objasnění úlohy cholesterolu a kardiovaskulárních rizikových faktorů (Vaughan 2000).

1. Ateroskleróza

Ateroskleróza je z etiopatogenetického hlediska považována za multifaktoriální onemocnění. Známe řadu faktorů, které se podílí nejen na vzniku ale i na její progresi, souhrnně je nazýváme rizikovými faktory (Dargel 1989).

1.1. Rizikové faktory

Udává se, že doposud bylo celkově identifikováno kolem 300 rizikových faktorů pro rozvoj aterosklerózy (Graham 2005). Můžeme je dělit podle několika hledisek, nejčastěji na rizikové faktory ovlivnitelné a neovlivnitelné.

Za hlavní **ovlivnitelné faktory** považujeme *kouření, arteriální hypertenzi, dyslipidémii, obezitu, diabetes mellitus, fyzickou inaktivitu.*

Mezi hlavní **neovlivnitelné faktory** patří *věk, mužské pohlaví a genetická predispozice*. Někteří autoři řadí do této skupiny i *faktory rasové (Muntner 2005).*

K dalším neméně důležitým rizikovým faktorům patří metabolický syndrom, zvýšená hladina triacylglycerolů, snížená hladina HDL cholesterolu, zvýšená hladina C-reaktivního proteinu (Hallan 2006), psychický stres a některá infekční agens.

1.1.1. Ovlivnitelné rizikové faktory

Kouření

Kouření je jedním z nejrozšířenějších rizikových faktorů aterosklerózy. Rizikové je nejen aktivní, ale jak bylo dokázáno na základě výsledků řady epidemiologických studií, i pasivní kouření (Hallan 2006). Kouření poškozuje cévní endotel a způsobuje endoteliální dysfunkci, snižuje HDL cholesterol, způsobuje hemodynamický stres, zvyšuje koagulační aktivitu, má proarytmogenní účinek, způsobuje relativní hypoxii (CO redukuje kapacitu Hb pro kyslík) a snižuje toleranci k fyzické zátěži. Zvyšuje tvorbu superoxidového radikálu, který inaktivuje NO a oxiduje LDL. Kouření je tedy komplexně působící agresivní rizikový faktor rozvoje aterosklerózy (Iacoviello 2001). Kouření je spojeno s vyšším výskytem lipidního proužkování u mladých lidí a podílí se také na progresi onemocnění zejména po 35. roku života (McGill 2001).

Hypertenze

Hypertenze zvyšuje produkci volných radikálů a také hladinu angiotenzinu II a endotelinu-1 a tím se podílí na vzniku aterosklerózy (Catena 2005). Způsobuje mechanické poškození endotelu. Zvýšení systolického krevního tlaku má větší vliv než zvýšení diastolického krevního tlaku. Hodnoty krevního tlaku nad 140/90 mmHg (u diabetiků 135/85 mmHg) vedou ke zvýšené koncentraci angiotenzinu II, který ovlivňuje aktivitu endotelových buněk, hladkých svalových buněk a makrofágů. V endotelových buňkách stimuluje tvorbu NF-κB (faktor spouštějící transkripci zánětlivých genů), dochází ke zvýšené adhezi leukocytů, expresi adhezních molekul a tvorbě superoxidu (reaguje s NO a způsobuje dysfunkci endotelu). Dále angiotenzin působí na růst a kontrakci cévních hladkých svalových buněk a zvyšuje jejich lipooxygenázovou aktivitu. Zvýšení této aktivity způsobuje vyšší produkci leukotrienů a lipoperoxidů s následnou oxidací LDL a tvorbou pěnových buněk. Léčba hypertenze snižuje incidenci cévních mozkových příhod, ICHS a srdečního selhávání (Shantaram 1999).

Porucha metabolismu lipidů (dyslipidémie)

Pro rozvoj aterosklerózy má hlavní úlohu vysoká hladina tzv. plazmatického cholesterolu, který můžeme nalézt v lipoproteinových částicích (VLDL a LDL) (Kita 2001). Jejich zvýšená hladina je často spojena se zvýšeným příjmem potravy a obezitou.

Hladinu LDL určuje rychlost vazby na LDL receptory v játrech. Vysoké hladiny LDL cholesterolu negativně ovlivňují endoteliální dysfunkci (zvyšují permeabilitu), způsobují vyšší migraci monocytů do subendoteliálních prostor, aktivaci endoteliálních buněk a následnou vyšší expresi adhezních molekul. Aterogenita LDL je způsobena především jejich schopností pronikat cévním endotelem a následně podléhat oxidativní modifikaci. Oxidativní modifikace vede k tomu, že tyto LDL nejsou rozpoznávány LDL receptory, ale scavengerovými receptory makrofágů, což vede k jejich kumulaci právě v makrofázích (Gudev 1996). Riziko vzniku a progrese aterosklerózy vzrůstá se zvýšenou koncentrací LDL v plazmě a s jejich klesající velikostí (Stampfer 1996). Snížení LDL o 1 % vede k poklesu rizika koronárních příhod o přibližně 2 % (Pedraza 1993). Aterogenita částic LDL klesá s jejich velikostí. Existuje pozitivní korelace mezi velikostí LDL

částic a rizikem infarktu myokardu (Strutt 2004). Malé LDL3 jsou často přítomny u nemocných s metabolickým syndromem a u nemocných s diabetus mellitus 2. typu (Gudev 1996).

Naproti tomu zvýšená hladina HDL cholesterolu je ochranným faktorem před vznikem aterosklerózy. HDL mobilizuje cholesterol z periferie do jater a žláz produkujících steroidy, navíc má i antioxidační vlastnosti a působí protizánětlivě. HDL pronikají do intimy, zajišťují reflux přebytečného cholesterolu, chrání LDL před oxidací, stimulují syntézu NO, inhibují adhezi monocytů, agregaci trombocytů, snižují krevní viskozitu a tlumí aktivitu t-PA a PAI-1. Proto je někdy označován jako „dobrý cholesterol" (Muntner 2005). Zvýšení HDL cholesterolu o 1 % snižuje riziko koronárních příhod o 2 – 3 %. Optimální hladina HDL cholesterolu je > 1,0 mmol/l. Zvýšení HDL cholesterolu nad 1,6 mmol/l je tzv. negativním rizikovým faktorem, který snižuje kardiovaskulární riziko (Coniglio 1997).

Dalším možným rizikovým faktorem je *lipoprotein(a).* Je to lipoproteinová částice podobná svou strukturou LDL částici. *Lipoprotein(a)* „soutěží" s plazminogenem o vazbu na plazmin, zasahuje tak do procesu fibrinolýzy, což vede k převaze trombogeneze nad fibrinolýzou (Iacoviello 2001). Některé studie prokázaly vztah mezi zvýšenou hladinou Lp(a) a rizikem rozvoje aterosklerózy (Dahlen 1997), zatímco jiné práce tuto teorii nepotvrdily (Ridker 1993).

Za samostatný rizikový faktor je považován zvýšený *apolipoprotein B* a snížený *apolipoprotein AI*. Jejich stanovení může mít někdy lepší předpovědní hodnotu, než měření LDL a HDL cholesterolu. Zvýšení apolipoproteinu B100 při normálním LDL cholesterolu může signalizovat zvýšený podíl malých LDL_3 v částic krvi (Graham 2005).

Diabetes mellitus, inzulínová rezistence, hyperinzulinémie

Riziko ICHS je u diabetiků 2 – 4x vyšší než u nediabetické populace (Bonnefont-Rousselot 2004). Není jasné, zdali hraje větší roli proces glykace proteinů (včetně LDL) a zvýšená tvorba vasokonstrikčních prostaglandinů, nebo doprovodná dyslipidémie, hypertenze a obezita.

Hyperglykémie podporuje glykaci proteinů, včetně lipoproteinů LDL (za vzniku tzv. AGEs). Tyto glykované LDL snáze podléhají oxidaci a jsou rozpoznávány

i scavangerovými receptory makrofágů, mohou aktivovat leukocyty a endoteliální buňky a navodit zánětlivý proces.

Inzulinová rezistence zvyšuje koncentraci VLDL, snižuje koncentraci HDL cholesterolu a zvyšuje výskyt arteriální hypertenze. Inzulin působí na cévní stěnu stimulací tvorby růstových faktorů, proliferací buněk hladkého svalstva, stimulací produkce pojivové tkáně, zvýšenou aktivitou LDL cholesterolu, zvýšenou tvorbou cholesterolu, zvýšenou tvorbou a sníženou regresí tukových proužků a zvýšením hladin plazmatického endotelinu-1 a inhibitoru aktivátoru plazminogenu 1 (PAI-1) (Shantaram 1999).

Obezita a nízká fyzická aktivita

Obezita představuje jeden z nejdéle známých a základních rizikových faktorů aterosklerózy. V poslední době přibývají přesvědčivé důkazy o tom, že rozhodujícím rizikovým faktorem není obezita jako taková, ale typ obezity.

Zvýšené riziko aterosklerózy představuje intraabdominální kumulace tuku. V současnosti se zdá být zcela zřetelné, že přítomnost intraabdominální obezity představuje na Body Mass Indexu (BMI) nezávislý a dokonce mnohem citlivější rizikový faktor rozvoje aterosklerózy či jejich klinických manifestací. Dobrým parametrem určujícím rozsah intraabdominální obezity je obvod pasu (normální hodnoty obvodu pasu: muž < 102 cm, žena < 88 cm). Intraabdnominálně uložené tukové buňky se vyznačují oproti jinde uloženým tukovým buňkám zvýšenou lipolytickou aktivitou, sníženou produkcí adiponektinu a zvýšenou produkcí řady prozánětlivých (TNF–α, IL6, CRP) a prokoagulačních faktorů (PAI-1). Není tak překvapením, že intraabdominální obezita je spojena s řadou proaterogenních jevů, jako je prozánětlivý stav, prokoagulační stav, hypertriglyceridémie a inzulínová rezistence s rozvojem hyperglykémie (Hallan 2006).

Studie prokázaly, že pravidelná fyzická aktivita snižuje riziko ICHS, kardiovaskulární i celkové mortality u mužů i u žen (Mehta 1998).

C-reaktivní protein

Jedná se o nespecifický, ale velmi citlivý marker zánětlivé reakce. Po stimulaci mediátory zánětu (IL-6, IL-8, TNF-α) je produkován nejen hepatocyty, ale také endoteliálními buňkami, hladkými svalovými buňkami a makrofágy.

Spolupodílí se na rozvoji aterosklerózy díky poškození fyziologické funkce endotelu, posílení prozánětlivého a prokoagulačního stavu (Egorova 2002). CRP je výborným predikčním ukazatelem, lepším než jiné markery zánětu (IL-6, TNF-alfa aj.)

V kardiologii se využívá metoda ultrasenzitivního měření CRP, tzv. hs -CRP (High sensitivity CRP) kde CRP slouží jako ukazatel rizika aterosklerózy (Hosseinsabet 2008).

Infekční agens

Uvažuje se o některých bakteriálních a virových patogenech - Chlamydia pneumoniae, Helicobacter pylori, Herpes simplex virus, Cytomegalovirus. Teoreticky mohou infekční agens ovlivnit vznik aterosklerózy řadou způsobů, jak lze demonstrovat na dopadu infekce gramnegativními bakteriemi - ovlivněním lipidového spektra (vzestup hladin triglyceridů, VLDL, pokles HDL), indukcí tvorby volných radikálů v cévní stěně (oxidace LDL, další poškození cévní stěny), indukcí prozánětlivých a prokoagulačních dějů (Laurila 1997).

1.1.2. Neovlivnitelné rizikové faktory

Genetická predispozice

Rozdílná četnost výskytu aterosklerotických tepenných změn byla potvrzena v 60. letech 20. století patologickou studií (International Atherosclerosis Project). Rovněž řada dalších studií prokázala „rodinný“ výskyt ICHS. Genetici analyzují celou řadu kandidátních genů pro rizikové faktory aterosklerózy, ale samozřejmě jednoduché vysvětlení není možné (Stolba 1992).

V současné době rozeznáváme ve vztahu k rozvoji aterosklerózy několik genových kategorií:

a) geny způsobující onemocnění spojené s rozvojem aterosklerózy, např. poruchy lipidového metabolismu, poruchy metabolismu homocysteinu,

b) geny způsobující aterosklerózu bez závislosti na jakémkoli jiném ději či rizikovém faktoru (tato oblast není zatím zcela prozkoumána, nalezení takovýchto genů by patrně představovalo revoluci v možnostech identifikace rizikových nemocných; v roce 2004 byl možná první z takovýchto genů popsán),

c) geny zodpovědné za náchylnost k rozvoji aterosklerózy – ohromná skupina genů a jejich variant s poměrně častým výskytem v populaci, jejich síla významu je ale v porovnání s významem klasických rizikových faktorů menší, patrně však v současnosti představuje nejvýznamnější klinickou roli, d) geny s aterosklerózou spojené – rovněž velká skupina genů, která je spojována s přítomností aterosklerózy, ale jejich přímý vztah k rozvoji aterosklerózy není doposud poznán či určen (Wang 2005).

Věk

Ateroskleróza je dlouhodobý proces, není proto divu, že pravděpodobnost jeho manifestace vzrůstá s věkem. Řada epidemiologických studií prokázala korelaci mezi vzrůstajícím věkem a vznikem aterosklerózy. Proto byla ateroskleróza považována za nemoc stáří. Za rizikový považujeme z hlediska ICHS věk 45 let a vyšší u muže a 55 let a vyšší u ženy (D'Agostino 2004).

Pohlaví

Hlavní příčinou většího výskytu aterosklerózy u mužů je rozdílné hormonální pozadí. Ženy jsou v premenopauzálním období chráněny estrogeny. Tyto ženské hormony mají vliv na složení lipidového spektra (nižší hladiny LDL a naopak vyšší hladiny HDL cholesterolu), dále ovlivňují inzulínovou rezistenci, hladinu cytokinů a funkci endotelu. V postmenopauzálním období takto tedy dochází ke zvýšenému výskytu kardiovaskulárních rizikových faktorů (Muntner 2005).

Další rizikové faktory

Z nejnovějších studií vyplynulo, že dalším rizikovým faktorem aterosklerózy je i obstrukční spánková apnoe. Její přítomnost je spojena prokazatelně s řadou proaterogenních jevů, jako je stimulace sympatiku, prokoagulační a prozánětlivý stav a endoteliální dysfunkce a další kardiovaskulární komplikace (Szaboova 2008).

1.2. Patofyziologie aterosklerózy

Aterosklerózu lze definovat jako chronické zánětlivé onemocnění, charakterizované endoteliální dysfunkcí s následným hromaděním lipidů, leukocytů, hladkých svalových buněka extracelulární matrix v intimě cév, což má za následek zužování cévního lumen s následnou redukcí až obstrukcí cévního průtoku (Brasen 1997).

Russell Ross jako jeden z prvních jasně definoval aterosklerózu jako zánětlivou chorobu, a jako počátek celého procesu vznik *endoteliální dysfunkce* (Ross 1999). Tímto termínem se označuje změna funkce (nikoliv morfologie) endotelu, která spouští „bludný kruh" změn vedoucích ke vzniku aterosklerotické léze.

1.2.1. Endoteliální dysfunkce

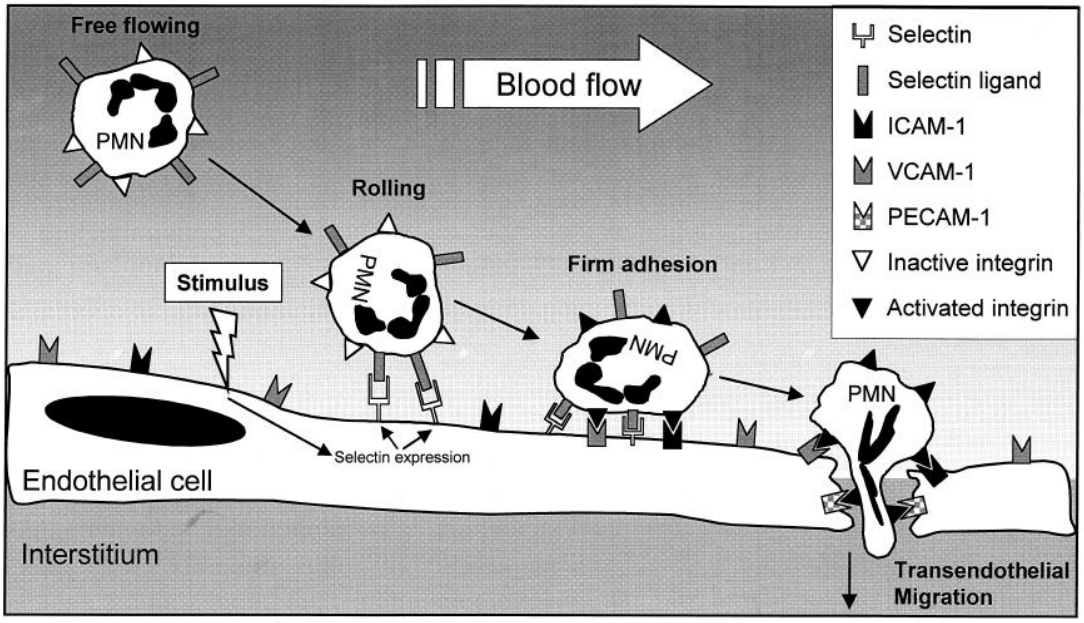
Endoteliální dysfunkce je prvním krokem aterogenního procesu. Vlivem dysfunkce endotelu a působení aterogenních faktorů dochází ke zvýšené kumulaci LDL v intimě cév (Corsini 1995). Tato akumulace není ani tak výsledkem zvýšené propustnosti endotelu, jako vazby lipoproteinů (zejména LDL) na makromolekuly extracelulární matrix v intimě (především proteoglykany). Takto vázané lipoproteiny mohou podléhat chemickým modifikacím – oxidaci a neenzymatické glykaci. Oxidace lipoproteinů je navíc usnadněna tím, že jsou tyto částice mimo dosah antioxidantů vyskytujících se v plasmě. K neenzymatické glykaci dochází nejčastěji u pacientů s diabetus mellitus s trvalou hyperglykémií (Giroux 1993). Oxidované (modifikované) LDL stimulují endotelové buňky k expresi buněčných adhezních molekul (VCAM-1, ICAM-1, E-selektin, P-selektin), k produkci chemotaktických faktorů pro monocyty (MCP-1), jakož i jejich receptorů. Kromě oxLDL a MCP-1 jsou dalšími chemoatraktanty, které indukují chemotaxi monocytů lipoprotein (a), degradovaný kolagen a elastin a cytokiny IL-1 a TNF-α. Všechny tyto faktory aktivují cirkulující monocyty a T lymfocyty, které začnou na svém povrchu exprimovat ve větším množství sacharidové (lektínové) receptory pro chemotaktické faktory a integriny (Springer 1995). Všechny tyto děje vedou k aktivaci a prostupu monocytů a T lymfocytů do intimy cév (Vestweber 1999).

Chemotaktický faktor MCP-1 hraje klíčovou roli v migraci leukocytů směrem k endotelu. Kromě endotelových buněk je syntetizován také hladkými

svalovými buňkami a makrofágy (viz obr. 1) (Oh 2001). Na povrchu leukocytů jsou přítomna vazebná místa pro selektiny. Po výše popsané aktivaci, dojde k expresi selektinů na povrchu endotelu, což způsobí první fázi v prostupu leukocytů do subendoteliálního prostoru, nazývanou tzv. „kutálením po endotelu“. Jedná se o slabou adhezní interakci.

Následná aktivace leukocytů prostřednictvím mediátorů zánětu umožní vytvořit pevnou vazbu mezi integriny na povrchu leukocytů a adhezními molekulami ICAM-1 a VCAM-1 exprimovanými endotelovými buňkami. Transmigraci leukocytů do subendoteliálního prostoru umožňuje endoteliální adhezní molekula PECAM-1, která se nachází v mezibuněčných spojích endoteliálních buněk (viz. obr.1).

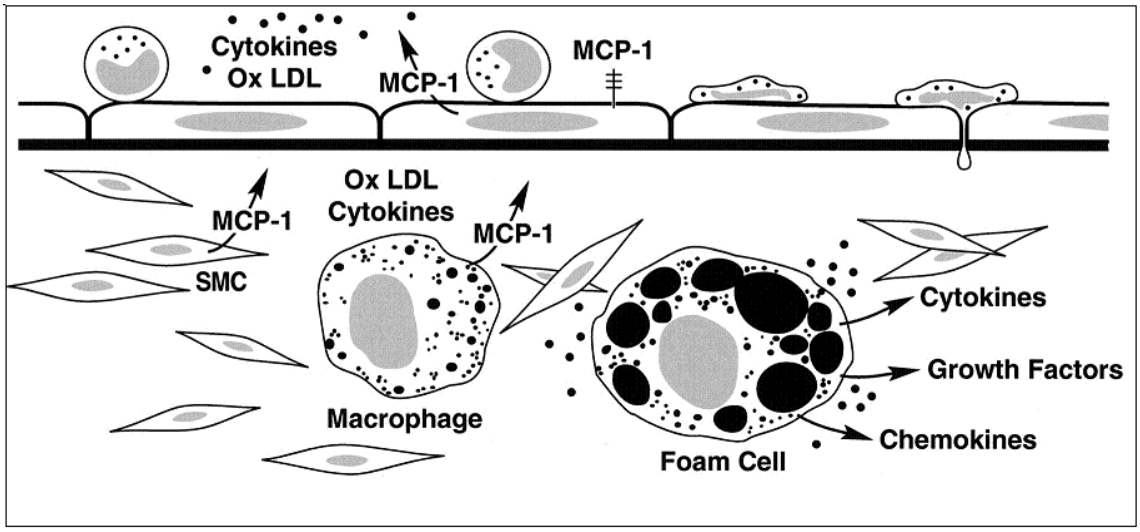
Obr. 1 Schéma transmigrace monocytů přes cévní endotel (Krieglstein 2001).



Prvními leukocyty, které se objevují v intimě cév jsou monocyty. Tyto monocyty jsou vystaveny působení růstových faktorů jako EDGF (endothelium-derived growth factor) nebo faktorům stimulujícím tvorbu kolonií jako např. M-CSF a díky nim dochází k jejich transformaci na makrofágy (Bjorkbacka 2008). Makrofágy pohlcují prostřednictvím svých „scavenger receptorů“ modifikované (oxidované) lipoproteiny, protože tyto lipoproteiny nemohou být katabolizovány cestou LDL receptorů. Scavanger receptory, které se nacházejí pouze na makrofázích a hladkých svalových buňkách, mají pro modifikované (oxidované) LDL přibližně 10x vyšší afinitu než klasické receptory pro LDL (Carr 2000). Jelikož

pohlcování oxLDL cestou scavangerových receptorů nepodléhá zpětnovazebné regulaci jako internalizace normálními LDL receptory, dochází k intracelulární akumulaci esterů cholesterolu a vzniku tzv. pěnových buněk (viz. obr. 2), jejich nahromaděním pak vzniká nejranější typ aterosklerotické léze nazvaný jako **tukové proužky (Krejsek 2005)**.

Obr. 2 Vznik pěnových buněk (foam cells) a produkce hlavních chemotaktických faktorů (Charo 2004).



1.2.2. Tukové proužky (fatty streaks)

Tukové proužky jsou nejčastější a u všech lidí přítomnou formou ateroskleróz. Vyskytují se běžně již v dětském věku a někdy je lze prokázat i u novorozenců. Jde o aterosklerotické léze, které nejsou ještě klinicky významné, to znamená, neprojeví se ischémií.

Makrofágy, které se podílejí na tvorbě tukových proužků, produkují množství látek, které ovlivňují další formování aterosklerotické léze. Jedním z nejvýznamnějších je chemokin MCP-1 (viz. obr. 2), který zesiluje chemotaxi makrofágů a T-lymfocytů a přispívá tak k jejich akumulaci v aterosklerotické lézi (Yla-Herttuala 1991). Dále jsou to růstové a zánětlivé faktory, které přispívají ke změně kontraktilního fenotypu hladkých svalových buněk na fenotyp syntetický a podporují proliferaci a migraci hladkých svalových buněk (Schwartz 1997), nebo faktory zvyšující expresi adhezních molekul VCAM-1 a ICAM-1. Různorodé cytokiny produkují i T-lymfocyty, které jsou v aterosklerotické lézi roztroušeny

mezi pěnovými buňkami, a tím se rovněž aktivně podílejí na progresi aterosklerotických lézí (Esaki 1997). V tomto případě je pravděpodobně nejvýznamnější INF-γ, který se spolupodílí na tvorbě pěnových buněk. Kromě toho inhibuje proliferaci hladkých svalových buněk a způsobuje tvorbu hydrolytických enzymů (metaloproteináz), které později narušují stabilitu aterosklerotického plátu (viz. níže) (Wouters 2005).

Další významnou buněčnou složkou, která se podílí na formování aterosklerotické léze, jsou hladké svalové buňky. Ty jsou za normálních okolností součástí médie cév, kde se podílejí na udržování cévního tonu. Zde se nacházejí v kontraktilním stavu. Růstové a zánětlivé faktory makrofágů a T lymfocytů způsobí jejich migraci z média do intimy a jejich transformaci z kontraktilního fenotypu na fenotyp syntetický. Syntetický fenotyp ztrácí schopnost kontrakce a získává schopnost proliferace a produkce cytokinů, růstových faktorů a tvoří také složky extracelulární matrix (kolagen, elastin, proteoglykany). Obsahuje rovněž již zmíněné scavengerové receptory a vychytáváním modifikovaných LDL přispívá k tvorbě pěnových buněk. Navíc bylo prokázáno, že tento fenotyp exprimuje také některé buněčné adhezní molekuly jako VCAM-1 a ICAM-1. Dále bylo zjištěno, že syntetický typ hladkých svalových buněk je náchylnější k apoptóze. Čím je apoptóza intenzivnější, tím je rychlejší i jejich proliferace. Proto se v místě aterosklerotických lézí nachází zvýšený počet odumřelých hladkých svalových buněk. Příčinou je i skutečnost, že syntetický fenotyp v porovnání s kontraktilním fenotypem obsahuje vyšší aktivitu kaspázy 3, klíčového enzymu v mechanismu apoptózy (Moiseeva 2001).

Kromě buněčné složky se v aterosklerotických lézích nachází i složka vláknitá, která je zastoupena především kolagenem. Ten je produkován nejen hladkými svalovými buňkami, ale z části také endoteliálními buňkami a fibroblasty. Syntéza kolagenu souvisí jak se změnou fenotypu, migrací a proliferací hladkých svalových buněk, tak s řadou lokálních i systémových činitelů (TGF-β, PDGF, endotelin-1, angiotensin II, IL-1, homocystein i mechanické napětí stimulují tvorbu kolagenu) (Lebrin 2005).

1.2.3. Pokročilé léze

Z výše uvedeného vyplývá, že po nahromadění makrofágů a T lymfocytů v intimě dochází v další fázi k transmigraci hladkých svalových buněk z medie do intimy a proliferaci extracelulární matrix, zejména kolagenu a vytvoření fibromuskulárního typu aterosklerotické léze.

I v tomto stádiu je ještě možná regrese a regenerace endotelových buněk v případě, že aterogenní faktor přestane působit. Výsledkem je pouze ztluštění intimy, která obsahuje jednu nebo dvě vrstvy myocytů, které se zde normálně nevyskytují. Pokud aterogenní faktor stále působí, proces se rozvíjí do dalších stádií (Linton 2003).

Makrofágy, které dále pohlcují lipoproteinové částice, se nyní soustřeďují ve střední části plátu. Na této kumulaci se makrofágy zřejmě mohou podílet také tím, že exprimují E-kadherin, který zajišťuje adhezi jednotlivých makrofágů k sobě (Bobryshev 1998). V této fázi dochází ke zvýšenému nahromadění volného cholesterolu, zatímco v počátečních stadiích makrofágy pohlcovaly především estery cholesterolu. Zvýšený poměr volný cholesterol/fosfolipidy zřejmě přispívá k odumírání makrofágů díky cytotoxickým účinkům volného cholesterolu (Wendelhag 1993).

Makrofágy, stejně tak jako hladké svalové buňky, v tomto stádiu podléhají zvýšené nekróze a apoptóze. Po odumření makrofágů dochází k extracelulárnímu nahromadění lipidů, uvolnění hydrolytických enzymů a zánětlivých látek, které vedou k vzniku nekrotického lipidového jádra. Hladké svalové buňky i nadále pokračují v migraci do intimy a v syntéze extracelulární matrix, zejména kolagenu, elastinu a proteoglykanů. Postupně tak dochází k vytvoření tzv. fibromuskulární čepičky na povrchu aterosklerotické léze. Je potvzeno, že hladké svalové buňky exprimují N-kadherin, který však neexprimují makrofágy. Právě homofilní interakce mezi N-kadheriny může vést ke specifické kumulaci hladkých svalových buněk u povrchu plátu (Yap 1997). Bylo zjištěno, že v aterosklerotických lézích se nacházejí proteiny vážící vápník. Je to například osteopontin, produkovaný hladkými svalovými buňkami pod vlivem růstových faktorů, nebo osteokalcin. Tím dochází v těchto nekrotických oblastech navíc k ukládání vápníku a mineralizaci (Palinski 2002).

Vytvořením lipidového jádra, zformováním fibromuskulární čepičky a ukládáním vápenatých iontů dochází k vytvoření pokročilé aterosklerotické léze, která se nazývá **ateromový plát**.

Pokročilé aterosklerotické léze jsou často příčinou klasických komplikací aterosklerózy, jako je stenóza a vznik trombóz, které pak omezují krevní průtok a vedou k orgánovým poškozením.

Z klinického hlediska můžeme aterosklerotické pláty rozdělit na stabilní a nestabilní**.**

Stabilní plát má nízký obsah tuků v jádře a pevnou fibromuskulární čepičku, proto nemá tendenci k ruptuře s vytvořením následné trombózy.

Nestabilní plát je bohatý na lipidy, jeho fibromuskulární čepička je tenká a dochází často k jeho prasknutí.

Zatímco stabilní pláty, které postupně „pouze" zužují cévní lumen, způsobují vznik typických námahových stenokardií při angině pectoris, trombóza, která provází nestabilní pláty, je zodpovědná za akutní koronární syndromy, nestabilní anginu pectoris a za vznik infarktu myokardu. Závažnost aterosklerotických plátů tedy nezávisí na jejich velikosti, ale zejména na jejich složení a charakteru. Nestabilní plát představuje typickou komplikovanou lézi (Badimon 1999)**.**

1.2.4. Komplikované léze

Tyto léze vznikají z ateromových plátů masivní kalcifikací a především těžkými degenerativními změnami (ulcerace, ruptura), které se pak stávají místem adherence trombocytů, agregace, trombózy a současné organizace trombu. Makroskopický vzhled komplikované léze odpovídá ateromovému plátu s následnými změnami v důsledku trombózy a přítomnosti erytrocytů (Stehbens 2002).

Ke vzniku trombu může dojít buď při rozrušení endotelu, nebo při ruptuře plátu. Malé poškození vede k odhalení kolagenu a tkáňového faktoru trombocytům a vede ke vzniku malých mikrotrombů. Ty obvykle nemají žádný klinický význam (Shah 2002). Při větším poškození endotelu dochází ke vzniku tzv. červeného trombu, který je bohatý na obsah trombocytů, erytrocytů a fibrinu. Tento trombus postupně uzavírá lumen cévy, může dojít i k jejímu úplnému uzávěru, nebo se

trombus může uvolnit a způsobit embolizaci. Navíc se v místě vzniku trombu rozvíjí zánětlivá reakce s hromaděním makrofágů a T-lymfocytů (Stehbens 2002).

2. Buněčné adhezní molekuly

Ateroskleróza může být chápána jako speciální druh chronického zánětu (Ross 1999). Pro průběh zánětu jsou důležité mezibuněčné interakce, včetně interakcí mezi leukocyty, mezi leukocyty a endotelem, leukocyty a hladkými svalovými buňkami a další. Proteiny, které zprostředkovávají tyto interakce, tzv. buněčné adhezní molekuly, jsou exprimované na povrchu všech tkání organismu. Podporují přilnavost buněk a také se účastní přenosu signálů mezi buňkami a podílejí se tak na interakci buněk s okolním prostředím. Adhezní molekuly se účastní řízení řady fyziologických dějů, jako je embryogeneze, růst buňky a její diferenciace, hojení ran nebo obnova tkání. Uplatňují se také při patologických procesech, jako je zánět, angiogeneze, trombóza a také při vzniku a rozvoji aterosklerózy (Joseph-Silverstein, 1998).

Podle strukturních vlastností můžeme adhezní molekuly rozdělit na 4 základní skupiny.

Selektiny

Integriny

Kadheriny

Imunoglobulinová skupina

2.1. Selektiny

Selektiny jsou tři a to E-, L- a P-selektin. Jsou to proteiny, které se účastní první fáze interakce lymfocytů s endotelem, kdy dochází k tzv. kutálení neboli rollingu (Vestweber 1999). E-selektin je exprimován na endoteliálních buňkách a zprostředkovává adhezi leukocytů na cévní endotel. L-selektin je exprimován na leukocytech (B, T lymfocyty, neutrolily, eosinofily) a také na nezralých erytrocytech a zajišťuje vazbu leukocytů na endotel v místě zánětu. P-selektin se nachází v alfa granulích destiček a Weibel-Paladeho tělíscích endoteliálních buněk a je důležitý při interakci mezi leukocyty, aktivovanými destičkami a endotelem (Tedder 1995). E-selektin je exprimován teprve po aktivaci endotelu zánětlivými

faktory jako TNF-α nebo interleukin-1 (Bevilacqua 1989) a P-selektin až po aktivaci histaminem, trombinem nebo např. oxidovanými LDL (Lehr 1991). Naproti tomu L-selektin je exprimován na leukocytech konstitučně. Všechny leukocyty včetně neutrofilů, T lymfocytů a monocytů využívají selektiny k prvnímu kontaktu a zachycení k endotelu (Vestweber 1999). Exprese P- a E-selektinu je zvýšená v aterosklerotických plátech (Johnson-Tidey 1994). Navíc bylo zjištěno, že P-selektin je společně s VCAM-1 exprimován endotelem ještě před akumulací makrofágů a T lymfocytů v intimě cév (Sakai 1997). Lze tedy říci, že E- a zejména P-selektin jsou markery časné aktivace endotelu a podílejí se na iniciační akumulaci makrofágů a T lymfocytů v intimě cév.

2.2. Integriny

Integriny jsou transmembránové glykoproteiny exprimované ve všech tkáních organismu (Howe 1998) a zejména na leukocytech a trombocytech (Lu 2008).

Z hlediska vztahu k ateroskleróze jsou významné 3 skupiny integrinů. Do skupiny β_1 integrinů patří tzv. VLA integriny. Jsou exprimovány na monocytech, T lymfocytech i trombocytech a váží většinou složky mezibuněčné hmoty jako kolagen, laminin, fibrinogen a fibronektin, ale také adhezní molekuly exprimované na endotelu. Druhou skupinu tvoří β_2 integriny, zvané též leukocytární integriny. Účastní se interakce jako komplementové receptory (Mareckova 1999). Třetí skupinu tvoří β_3 integriny, které se uplatňují hlavně při interakci trombocytů se složkami mezibuněčné hmoty a hrají tak zásadní roli při zachycování destiček v místě vaskulárního poškození (Joseph-Silverstein 1998).

Integriny hrají zásadní roli při migraci leukocytů z krve do tkání a to zejména při rolování a vytváření pevné vazby k endotelu (Alon 1995). Vazba integrinů na ligand vede také k přenosu řady intracelulárních signálů, které pak mohou ovlivňovat interakce mezi buňkami (McGilvray 1997). Integriny jsou zásadní pro vytvoření pevné a stabilní vazby leukocytů k endotelu v místě zánětlivé reakce.

2.3. Kadheriny

Kadheriny jsou transmembránové glykoproteiny, které zprostředkovávají adhezi buněk a jsou závislé na přítomnosti Ca2+ iontů. Kadheriny jsou hlavními strukturálními glykoproteiny, které tvoří adherentní mezibuněčné spoje nazývané zonula adherens (adherens junctions) (Dejana 1997).

Kadheriny mají dvě hlavní funkce. Jsou zodpovědné za mezibuněčnou adhezi a podílejí se na přenosu signálu mezi buňkami (Price 1999).

Dnes je popsáno velké množství kadherinů, které se liší strukturou svých domén a také místem svého výskytu. Většina názvů kadherinů je odvozena od místa jejich výskytu, ale různé tkáně exprimují různé kadheriny (Behrens 1999). Ve vztahu k ateroskleróze mají největší význam epiteliální (E)-kadherin a vaskulární-endoteliální (VE)-kadherin.

E-kadherin je hlavním kadherinem exprimovaným epiteliálními buňkami. Z hlediska funkce je nepostradatelný pro normální embryogenezi a morfogenezi mnohých tkání (Nachtigal 2001). Exprese E-kadherinu byla zjištěna na pěnových buňkách v lidských aterosklerotických lézích. Vypadá to, že E-kadherin se podílí na agregaci pěnových buněk a tím se podílí i na formování lipidového jádra (Bobryshev 1998)

VE-kadherin je kadherin specifický pro cévní endotel. Je hlavní adhezní molekulou mezibuněčných spojů – zonula adherens (Dejana 1999). VE-kadherin je nutný pro normální vaskulogenezi, angiogenezi a je velmi důležitý pro udržení endoteliální integrity a permeability (Dejana 1999). Porucha exprese a funkce VE-kadherinu vede ke vzniku endoteliální dysfunkce (Nachtigal 2001). VE-kadherin je exprimován v lidských aterosklerotických lézích, kde se podílí na neovaskularizaci kapilár, které jsou důležité pro rozvoj lokální zánětlivé reakce (Bobryshev 1998).

2.4. Imunoglobulinová skupina adhezních molekul

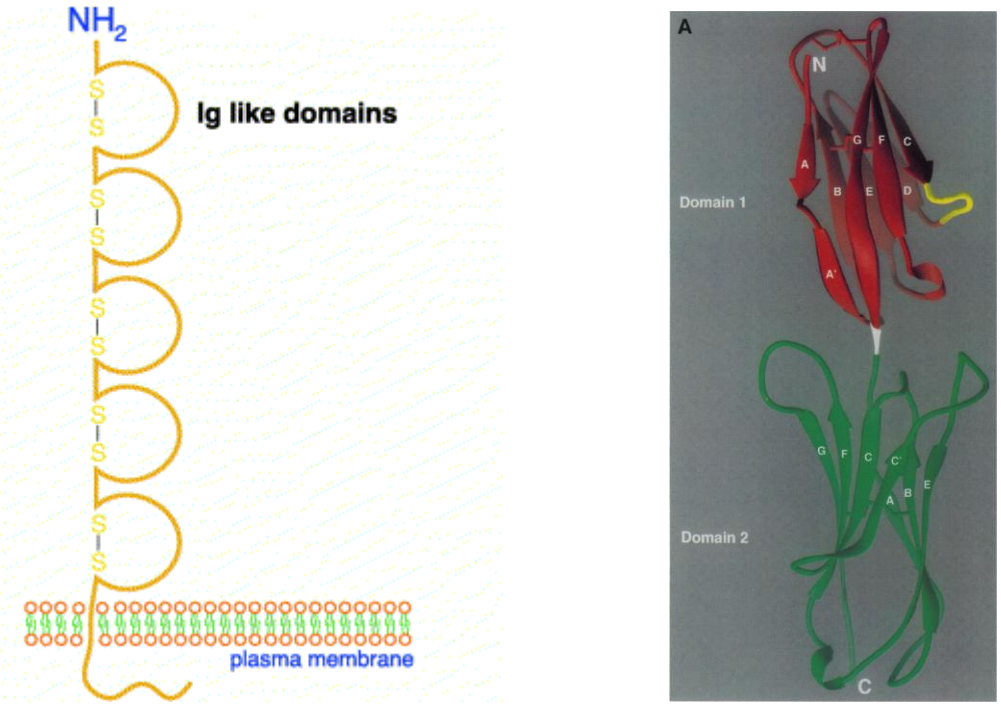
Tato skupina zahrnuje celou řadu povrchových buněčných molekul. Jde o glykoproteiny tvořené opakujícími se Ig doménami z beta řetězců, které zprostředkovávají jak hemofilní (vazba adhezní molekuly v jedné buňce na stejnou molekulu ve druhé) tak heterofilní interakce (vazba adhezní molekuly v jedné buňce na neidentickou molekulu ve druhé buňce) (Mareckova 1999). Patří sem celá řada adhezních molekul jako antigenně specifické receptory T a B lymfocytů

TCR, BCR, koreceptory T lymfocytů CD4 a CD8, které jsou důležité pro jejich správnou funkci při imunitních reakcích. Z hlediska vztahu k ateroskleróze jsou nejvýznamnějšími zástupci VCAM-1 (vascular cell adhesion molecule-1), ICAM-1 (intracellular cell adhesion molecule-1) a PECAM-1 (platelet-endothelial cell adhesion molecule-1).

Struktura, funkce a exprese VCAM-1 a ICAM-1

Z hlediska struktury jsou obě adhezní molekuly transmembránové glykoproteiny obsahující N-konec, sérii Ig domén, transmembránovou oblast a cytoplazmatický konec (obr. 4).

Obr. 3 Struktura ICAM-1 podle Price DT et al a VCAM-1 podle Wang JH et al. (Price 1999, Wang 2001).



VCAM-1 má 7 extracelulárních domén, přičemž domény 1 a 4 jsou specifická vazebná místa pro vazbu $\alpha_4\beta_1$ integrinu (VLA-4) a někdy pro vazbu $\alpha_4\beta_7$ integrinu (Springer 1994). ICAM-1 má 5 extracelulárních domén, přičemž domény 1 a 3 jsou specifická vazebná místa pro vazbu $\alpha_L\beta_2$ integrinu (LFA-1), respektive pro vazbu $\alpha_M\beta_2$ integrinu (Mac-1), které jsou exprimovány na leukocytech (Jang 1994).

ICAM-1 a VCAM-1 se podílejí na stabilizaci vazby leukocytů k endotelu a podílí se tedy na jejich diapedezi (Cybulsky 1999).

ICAM-1 i VCAM-1 jsou exprimovány endoteliálními buňkami, makrofágy a hladkými svalovými buňkami (Jang 1994). Studie na králících a myších prokázaly, že VCAM-1 je endoteliálními buňkami exprimován ještě před hromaděním makrofágů a T lymfocytů a to v oblastech, které jsou predispoziční ke vzniku lézí, přičemž lokalizace těchto míst je často ovlivněna hemodynamickými vlastnostmi především shear stresem. ICAM-1 je exprimován ve stejných oblastech jako VCAM-1, ale exprese ICAM-1 je pozorována i v oblastech s nízkou pravděpodobností výskytu aterosklerotických lézí. U malých aterosklerotických lézí je VCAM-1 i ICAM-1 exprimován především endoteliálními buňkami, přičemž VCAM-1 je exprimován i hladkými svalovými buňkami, které přiléhají k aterosklerotické lézi. U pokročilejších aterosklerotických lézí je VCAM-1 i ICAM-1 exprimován většinou buněk, které se nacházejí v intimě cév. Přesto se ukazuje, že VCAM-1 je exprimován především v oblastech výskytu lézí, zatímco ICAM-1 je exprimován endoteliálními buňkami i mimo aterosklerotickou lézi (Iiyama 1999).

Exprese těchto adhezních molekul je ovlivňována řadou faktorů, které se uplatňují i v patogenezi ateroskleróz. Hypercholesterolemie, oxidované LDL a diabetes zvyšují expresi jak ICAM-1 (Bevilacqua 1989), tak VCAM-1 (Khan 1995), (Vlassara 1995). Také kouření, hyperhomocysteinemie, hemodynamický stres (nízký shear stress) zvyšují expresi VCAM-1 a ICAM-1 (Powell 1998). Exprese obou těchto molekul je také indukována zánětlivými cytokiny jako TNF-α nebo IL-1 (Marui 1993). V séru můžeme detekovat rozpustnou isoformu ICAM-1 za fyziologických podmínek, ve významně zvýšeném množství pak při různých patologických dějích (Henninger 1997).

PECAM-1 (platelet endothelial cell adhesion molecule)

Další zástupce adhezních molekul imunoglobulinové rodiny. PECAM-1 je glykoprotein, který má 6 extracelulárních Ig domén, transmembránovou oblast a cytoplazmatický konec (Muller 1993).

PECAM-1 je exprimován především na endoteliálních buňkách v místě intercelulárních spojů, na trombocytech a většině leukocytů. Jelikož exprese

PECAM není závislá na stimulaci cytokiny, hustota těchto molekul se používá k odhadu plochy cévního řečiště (Watt 1995).

PECAM-1 hraje důležitou roli při vaskulogenezi, angiogenezi a významně se podílí na prostupu leukocytů do subendoteliáních prostorů (Newman 1990).

Pokusy *in vivo*, které byly zaměřeny na chování leukocytů, vedly k vytvoření modelu, který předpokládá tři postupné fáze při pohybu leukocytů cévním endotelem: rolování („kutálení po endotelu"), pevnou adhezi (přilnutí) a vycestování z cévního řečiště – transendoteliální migraci (Krieglstein 2001).

Rolování (slabé adhezní interakce) umožňují selektiny, pro které mají leukocyty na svém povrchu příslušné ligandy. Přestože na leukocytech můžeme nalézt i jiné adhezní molekuly (například VLA-4, VCAM-1, MAdCAM-1 a zástupce ze skupiny β_1 integrinů), jejich kvantitativní význam v procesu rolování zůstává zatím nejasný (Jang 1994). Adherované leukocyty jsou dále vystaveny působení nízkých koncentrací chemoatraktantů/mediátorů zánětu, které způsobí jejich aktivaci a následně vyvolají integrin-Imunoglobulin dependentní vazbu leukocytů k povrchu endotelu. Jedná se již o pevnou adhezní interakci. Aktivace leukocytů je rovněž spojená se zvýšenou aktivitou integrinů, která může být vyvolána chemokiny, bakteriálními peptidy, PAF (faktor aktivující destičky) a leukotrieny B_4 (Cybulsky 1999). Transendoteliální migrace začíná pohybem takto přilnutých leukocytů směrem k mezibuněčným spojům.

K vytvoření infiltrace velkého počtu leukocytů v zanícené tkáni je nezbytná vysoká hustota endoteliálních adhezních molekul. Ta je udržována pouze syntézou proteinů *de novo*. Syntézu různých endoteliálních adhezních molekul (zahrnujících ICAM-1, VCAM-1, MAdCAM-1, E-selektin a P-selektin) indukuje velké množství rozmanitých bakteriálních toxinů, cytokinů a oxidantů (Cybulsky 1999).

3. Endoglin - CD 105

Endoglin je homodimerní transmembránový glykoprotein skládající se ze dvou podjednotek o velikosti 95 kDa propojených disulfidickým můstkem (ten Dijke 2008). Lidský endoglin má tři části - extracelulární doménu o velikosti 561 aminokyselin, jednoduchou transmembránovou část a cytoplasmatickou část (Bellon 1993). U člověka byly popsány dvě izoformy (L a S), které se liší

cytoplasmatickou částí, stejně tak existují podobné dvě izoformy L- a S-endoglin také v myších tkáních (Perez-Gomez 2005).

Endoglin byl prvně identifikován na pre-B leukemické buněčné linii (van Laake 2006). Hlavní tkání, kde je exprimován, jsou buňky cévního endotelu. Kromě těchto buněk ho v menší míře exprimují i další buňky - cévní hladké svalové buňky (Adam 1998), fibroblasty (St-Jacques 1994), makrofágy (Lastres 1992), leukemické buňky pre – B a myelomonocytárního původu (Perez-Gomez 2005) a prekursory erytrocytů (Buhring 1991). Syncytiotrofoblast a terminální placenta obsahují vysokou hladinu CD105 (St-Jacques 1994).

Normální T, B lymfocyty a nestimulované monocyty endoglin neexprimují, ale zvýšená exprese je sledována na aktivovaných makrofázích (Lastres 1992). Stejně tak je vysoce exprimován na endoteliálních buňkách ve tkáních, ve kterých probíhá angiogeneze, jako jsou hojící se rány, infarkty a celá řada nádorů (Guo 2004), také v hladkých svalových buňkách cév během zánětu a poranění (Adam 1998). Zvýšené sérové hladiny endoglinu se objevují při ateroskleróze (Blann 1996). V lidských aterosklerotických lézích byla sledována zvýšená exprese endoglinu hlavně v hladkých svalových buňkách, makrofázích a endoteliálních buňkách v oblastech lézí, zatímco v hladkosvalových buňkách zdravé arteriální stěny nebyl endoglin detekován (Conley 2000, Piao 2006).

Endoglin je součástí TGF β-receptorového komplexu přítomného v endotelových buňkách. TGF-β je rodina polypeptidů, které různým způsobem ovlivňují růst, diferenciaci, adhezi a apoptózu buněk a tvorbu mezibuněčné hmoty. Pro rozvoj aterosklerózy je významný zejména TGF–β1. Je to pleiotropní růstový faktor, který má významné protizánětlivé účinky. Tyto účinky byly ověřeny na endoteliáních, hladkých svalových buňkách, ale i makrofázích a T-lymfocytech (Lebrin 2005).

TGF–β se na buněčném povrchu váže na receptor pro TGF-β typu II, dochází k fosforylaci – aktivaci receptoru pro TGF-β typu I. Tento aktivovaný komplex fosforyluje – aktivuje nitrobuněčné posly tzv. Smad proteiny, jejichž prostřednictvím je informace přenesena až do jádra, kde dochází k ovlivnění transkripce. Podle studií *in vitro* se zdá, že protizánětlivé účinky TGF-β1 jsou zprostředkované především proteiny Smad 3 a Smad 2 (Feinberg 2005). Endoglin, někdy označován jako receptor pro TGF-β typu III, nemá vnitřní kinázovou

aktivitu, ale ovlivňuje TGF-β signalizaci. Jak? To není ještě zcela úplně jasné. Je prokázáno, že endoglin se váže na TGF-β1 a TGF-β3, ne však na TGF-β2 (Cheifetz 1992, Bellon 1993).

Zvýšená exprese endoglinu v myších fibroblastech byla spojována se sníženou migrací a pozměněnou morfologií buněk (Guerrero-Esteo 1999). V L6E9 myoblastech zvýšená exprese endoglinu snížila citlivost buněk na TGF-β1, což způsobilo inhibici růstu těchto buněk (Letamendia 1998). Tyto a další výsledky (Lastres 1996) naznačují, že endoglin ovlivňuje odpověď buněk na TGF-β1, přičemž se předpokládá, že usnadňuje vazbu TGF-β1 na receptory.

Endoglin hraje také velmi důležitou úlohu v udržení cévní homeostázy. Mutace genu pro endoglin je spojena s rozvojem hereditární hemoragické telaengiektázie (HHT) typu I (McAllister 1994).

Nedávné výsledky ukazují, že endoglin ovlivňuje také vazodilataci prostřednictvím oxidu dusnatého (Jerkic 2004). Oxid dusnatý odvozený od endoteliální NO syntázy je důležitý endogenní vazodilatační faktor, který reguluje tonus v krevním řečišti a udržuje anti-trombotické, anti-proliferativní a anti-apoptotické prostředí v stěně cév (Sessa 2004). Endoglin *in vitro* zvyšuje expresi endoteliální NO syntázy (eNOS) ovlivněním hladiny Smad 2 (Santibanez 2007).

4. Statiny

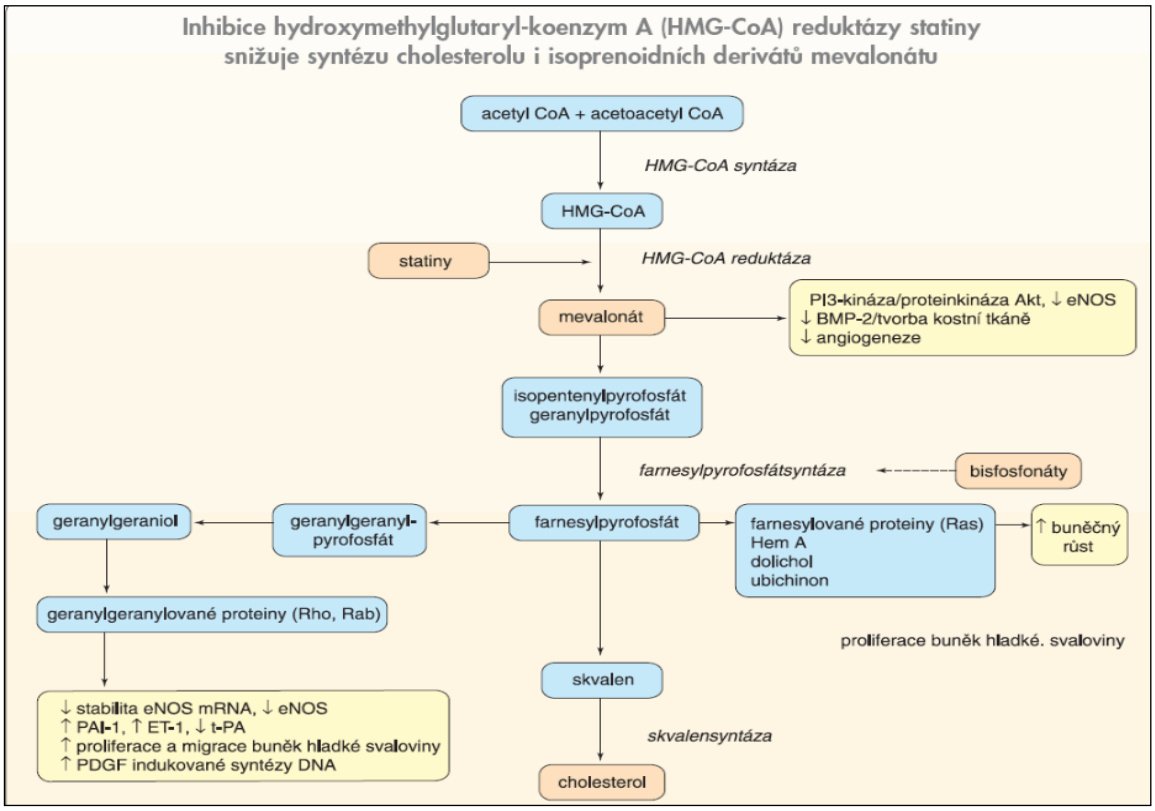
Statiny neboli kompetitivní inhibitory 3-hydroxy-3-methyl-glutaryl-koenzym A-reduktázy, patří v současné době mezi nejúčinnější a celosvětově nejpoužívanější hypolipidemika s příznivými účinky nejen na hladiny sérových lipidů, ale i na kardiovaskulární a celkovou mortalitu (Vaughan 2000). Randomizované klinické studie prokázaly, že léčba statiny snižuje výskyt infarktu myokardu a ischemické cévní mozkové příhody v primární i sekundární prevenci u pacientů s různě závažnou hypercholesterolémií.

Statiny snižují především hladiny celkového cholesterolu a LDL cholesterolu. Řada experimentálních i klinických studií ukazuje, že prospěch z užívání statinů je dán nejen jejich schopností snižovat hladiny plazmatických lipidů, ale i jejich četnými extralipidovými účinky, které jsou na snížení plazmatických lipidů nezávislé (Arnaud 2005).

4.1. Mechanizmus účinku statinů a jejich lipidové účinky

Statiny inhibují klíčový enzym syntézy cholesterolu. Limitující reakcí endogenní biosyntézy cholesterolu je převedení aktivované kyseliny 3-hydroxy-3-methylglutarové (HMG-CoA) na kyselinu mevalonovou (viz. obr. 4). Tuto reakci katalyzuje HMG-CoA-reduktáza lokalizovaná v hladkém endoplazmatickém retikulu jaterních buněk, dá se však prokázat i v jiných tkáních. Statiny obsahují ve své molekule skupiny strukturálně podobné substrátu, proto mohou tento enzym kompetitivně inhibovat. Jeho inhibicí dochází ke snížení nitrobuněčné syntézy cholesterolu a buňka se dostává do situace deficitu cholesterolu. Ten pak vede ke zvýšené transkripci LDL receptorového genu a zvýšené expresi LDL receptorů na buněčné membráně všech buněk, především však hepatocytů. Zvýšená syntéza a zvýšení aktivity LDL receptorů vedou k urychlenému vychytávání LDL z plazmy (Tonolo 2003). Snížení LDL cholesterolu může dosáhnout až 40 %. Navíc dochází obvykle k mírnému zvýšení HDL cholesterolu (až o 15 %) a snížení koncentrace triacylglycerolů až o 25 %. Tento mechanizmus není zcela znám. Předpokládá se snížení syntézy VLDL v játrech. Dalším možným mechanizmem je zvýšení clearance a odbourávání VLDL cestou LDL receptorů. To je umožněno přítomností apolipoproteinu E na částicích VLDL, který je schopen se vázat na LDL receptor. Nepřímo jsou tak ovlivněny i hladiny HDL. Teprve nedávno bylo prokázáno, že statiny jsou schopny snižovat i koncentraci „malých denzních LDL částic“ (Duriez 2003).

Obr. 4 Schéma biosyntézy cholesterolu - (Tonolo 2003)



4.2. Pleiotropní (extralipidové) účinky statinů

Kromě příznivého účinku na hladiny krevních lipidů mají statiny další, na lipidech zcela nezávislé vlastnosti, které se podílejí na snižování kardiovaskulárního rizika. Jsou označovány jako tzv. pleiotropní účinky (Vaughan 1996). Pleiotropní účinky vyplývají z inhibice HMG-CoA-reduktázy. Kyselina mevalonová je prekurzorem nejen cholesterolu, ale i množství nesteroidních isoprenoidních sloučenin, které se účastní procesů buněčného metabolismu a mezibuněčné komunikace (viz. obr. 5). Tyto látky mají rozmanité funkce – podílejí se na regulaci buněčného růstu, expresi NO-syntázy, migraci hladkých svalových buněk, ovlivnění trombogenní aktivity a mnoha dalších dějích. Mezi nejdůležitější „nelipidové“ účinky statinů patří zlepšení endoteliální funkce, antioxidační, protizánětlivé, antiproliferační a antitrombogenní účinky (Mehta 2003).

Zlepšení endoteliální dysfunkce

Charakteristickým příznakem endoteliální dysfunkce je poškozená syntéza, uvolňování NO z endotelu a také jeho aktivita. Statiny zlepšují endoteliální

dysfunkci zčásti snižováním LDL cholesterolu, specifičtěji však působí na expresi a aktivitu endoteliální NO syntázy (eNOS), enzymu, který katalyzuje vznik NO z L-argininu, čímž zvyšují dostupnost NO. Předpokládá se, že statiny takto kompenzují nedostatečnou tvorbu NO v aterosklerotických lézích a působí tak proti progresi aterosklerózy (Vaughan 1996). Tlumí i uvolňování a aktivitu vazokonstrikčních látek (například endotelinu a angiotenzinu II) (Auer 2002).

Antioxidační účinky

Uvažuje se minimálně o 4 mechanismech antioxidačního působení: 1) jelikož dochází ke snížení koncentrace LDL cholesterolu, vzniká tím pádem i méně oxLDL, 2) inhibicí NADH oxidáz dochází ke snížení tvorby vaskulárních a endoteliálních superoxidů; vzniku oxLDL brání i udržování aktivity vnitřních antioxidačních systémů (například superoxid dismutázy), 3) přímou vazbou statinů na fosfolipidy lipoproteinové částice dochází k ochraně lipidového jádra před volnými radikály, 4) silná antioxidační aktivita statinových metabolitů (např. u atorvastatinu a fluvastatinu) rovněž přispívá k ochraně před oxidačními procesy (Calabro 2005).

Protizánětlivé účinky

Zánětlivý proces probíhající v cévním endotelu zvyšuje riziko kardiovaskulárních příhod a tvoří významný faktor rozvoje metabolického syndromu (Albert 2001). Bylo zjištěno, že statiny mají protizánětlivé účinky, snižují hladinu C-reaktivního proteinu), jehož hladina je zvýšená i při rozvoji aterosklerózy. Významné je i snižování exprese mnoha adhezních a chemotaktických molekul a inhibice aktivity integritu (Arnaud 2005).

Statiny a trombóza

Antitrombotické působení statinů spočívá v příznivém ovlivnění agregace destiček, krevní viskozity, hladin tkáňového faktoru a jeho přirozeného inhibitoru, fibrinogenu, tkáňového aktivátoru plazminogenu (tPA) a jeho přirozeného inhibitoru (PAI-1) a rovněž lipoproteinu (a). Dochází k inhibici buněčné exprese tkáňového faktoru v mikrofázích (Palinski 2002), k normalizaci tvorby trombinu u pacientů s hypercholesterolémií a ke snížení agregace destiček. Snížení agregace

destiček má pravděpodobně vztah k redukci obsahu cholesterolu v jejich buněčných membránách (Laufs 2003).

Stabilizace aterosklerotického plátu

Statiny přispívají ke stabilizaci aterosklerotického plátu více mechanismy. Snižují hladiny oxidovaného LDL cholesterolu a jeho vychytávání makrofágy, inhibují matrixové metaloproteinázy, které se podílejí na destabilizaci aterosklerotických plátů, zvýšují obsah kolagenu v plátech (Comparato 2001).

Efekt na hladké svalové buňky

Pomocí statinů dochází též k inhibici proliferace a migrace hladkých svalových buněk v cévní stěně. Zatímco většina autorů považuje inhibici proliferace myocytů za pozitivní efekt léčby, který působí protiaterogenně, objevují se i opačné názory, které považují proliferaci myocytů za významný faktor při stabilizaci plátů (Vaughan 2000).

Další příznivé účinky statinů

Výzkumy potvrdily, že statiny inhibují růst nádorových buněk. Bylo zjištěno, že většina nádorových buněk vykazuje zvýšenou aktivitu HMG-CoA reduktázy, proto se předpokládá, že selektivní inhibice tohoto enzymu by mohla vést k novým možnostem léčby rakoviny.

Statiny se uplatňují i v kostním metabolismu. Dochází ke snížení aktivity osteoklastů a snížení kostní resorpce, naopak se zvyšuje novotvorba kostní hmoty. Pravděpodobně proto je podávání statinů spojené se sníženým rizikem vzniku kostních zlomenin u pacientů starších 50 let.

Použití statinů v jiných než hypolipidemických indikacích však zatím brání nedostatek randomizovaných klinických studií, takže obecně platí názor, že extralipidové účinky statinů efektivně doplňují jejich přímý účinek na hladinu lipidů (Wierzbicki 2003).

I když je působení pleiotropních účinků jistě významné, je třeba zdůraznit, že jejich role je ve srovnání s hypolipidemickým efektem pravděpodobně méně důležitá. Za převážnou většinu pozitivních kardiovaskulárních účinků statinů stojí jejich vliv na LDL-cholesterol.

4.3. Atorvastatin

Atorvastatin patří v současné době k nejvíce užívaným statinům. Je podáván ve formě aktivní látky. Po perorálním podání se snadno a rychle vstřebává, maximálních plazmatických koncentrací je dosaženo přibližně za 2,5 hodiny. Z důvodu vysokého first-pass metabolismu v játrech je biologická dostupnost atorvastatinu pouze 12 %. Přibližně z 98 % se váže na plasmatické bílkoviny. Je metabolizován cytochromem P450 3A4 na biologicky aktivní metabolity, které přispívají k inhibici HMG-CoA reduktázy asi ze 70 %. Po hepatální a extrahepatální metabolizaci jsou atorvastatin a jeho metabolity primárně eliminovány žlučí, přičemž nedochází k enterohepatální cirkulaci. Jeho eliminační poločas je v průměru 14 hodin, ale díky přítomnosti aktivních metabolitů přetrvává inhibiční účinek na HMG-CoA reduktázu asi 24 hodin (Regazzi 1994).

Studie prokázaly, že u pacientů s hypercholesterolémií snižuje atorvastatin hladinu LDL cholesterolu, celkového cholesterolu a především triacylglycelů efektivněji než ostatní statiny. Již při obvyklých dávkách dochází k poklesu koncentrace triglyceridů okolo 20 %. Maximální snížení LDL cholesterolu může dosáhnout až 90 %. Naopak zvýšení hladiny HDL cholesterolu není tak významné (5 – 15 %). Pozitivním jevem je změna velikosti LDL částic od malých denzních aterogenních částic k částicím větším (Desager 1996).

Nežádoucí účinky atorvastatinu jsou mírné a mají přechodný charakter, lék je obecně dobře snášen. Nejčastěji se vyskytují zažívací potíže, bolesti hlavy a svalů, kopřivka nebo ospalost. Stejně tak jako u jiných statinů, i po podání atorvastatinu bylo popsáno zvýšení hladin jaterních transamináz. Výskyt závažné rabdomyolýzy spojované s léčbou statinů, která může vést až k ledvinnému selhání, však zatím u atorvastatinu nebyl prokázán.

I když studie na zvířatech nepotvrdily teratogenní účinky atorvastatinu, všechny statiny jsou kontraindikovány v těhotenství a u kojících matek (Sinzinger 2002).

5. Zvířecí modely

Pro studium patogeneze a potenciální léčby ateroskleróza se používalo a používá několik živočišných druhů. První důkaz o studiu experimentální aterosklerózy byl uveřejněn již v roce 1908, při podávání diety bohaté na živočišné proteiny (maso, mléko, vejce) králíkovi, došlo k ztluštění jeho cévní intimy, které bylo způsobeno velkými buňkami v aortě (Jawien 2004).

Později se používali například primáti, prasata, příležitostně také křečci a holubi. Ukázalo se ovšem, že tyto modely nejsou vhodné. Rovněž potkani a psi nejsou vhodnými modely, protože k tvorbě lézí, které v jejich cévách nevznikají spontánně, vyžadují vysoké nároky na dietu.

Dobře známým a poměrně hojně používaným zvířecím modelem je **králík – Novozélandský bílý**. Králík se z hlediska lipoproteinového metabolismu podobá v několika ohledech člověku. Má například podobné složení lipoproetinů obsahujících apolipoprotein B (Chapman 1980), produkuje játry VLDL obsahující apoB_{100} (Greeve 1993), má podobnou aktivitu plasmatické CETP, a vysokou rychlost absorpce dietárního cholesterolu (Yang 1998).

U králíků se ateroskleróza rovněž nerozvíjí spontánně, ale po cholesterolové dietě vznikají léze v poměrně krátkém čase (Ito 1994). Léze u králíka jsou více bohaté na tuky a makrofágy, než léze v lidských cévách a na rozdíl od člověka se u králíka vyskytují výjimečně vysoké hodnoty plazmatického cholesterolu. I lokalizace lézí je jiná než u člověka. Na rozdíl od člověka, kde téměř vždy vznikají léze v oblasti koronárních arterií a břišní aorty, u králíka je to zejména v oblasti aortálního oblouku a hrudní aorty (Yang 1998).

Pokud chceme u králíka navodit pokročilejší aterosklerotické léze, je nutné mechanické poškození. Jednou z neméně důležitých nevýhod je také nedostupnost komerčně vyráběných chemikálií určených na zpracování a analýzu králičích vzorků.

WHHL králík - **Watanabe heritable hyperlipidemic** je LDL receptor deficientní kmen králíků, který představuje model familiární hypercholesterolémie. Vyvíjejí se u nich spontánní aterosklerotické léze, které jsou morfologicky podobné lidským lézím (Ito 1994). Pro studium vlivu jednotlivých apolipoproteinů na metabolismus lipidů byli vyvinuti WHHL apoA-1 transgenní králíci a NZW apoB-100 trangenní králíci. WHHL apoA-1 králíci jsou modifikováni

pro expresi lidského apoA-1, dochází u nich ke zvýšení hladiny HDL cholesterolu bez změny hladiny LDL cholesterolu a také ke snížení velikosti aterosklerotických plátů (Duverger 1996). U NZW apoB-100 trangenních králíků se objevuje až 3x zvýšená hladina celkového cholesterolu a triacylglycerolů a naopak snížená hladina HDL cholesterolu (Fan 1995).

Až do roku 1992 byla převážná většina výzkumu v oblasti aterosklerózy prováděna na modelu králíka, v menší míře pak na modelech prasete a primátů. Během výzkumů se získaly neocenitelné poznatky. Na prasečím modelu bylo odhaleno, že jedním z primárních dějů v procesu aterosklerózy je infiltrace monocyty (Hotta 1999). Studie na opicích a králících vedly k objasnění buněčných procesů v procesu vzniku a rozvoje aterosklerotických lézí.

Dnes se pro studium aterosklerózy *in vivo* hojně využívají **myší** modely.

5.1. Myší model aterosklerózy

Myší model pomohl díky své velikosti překonat množství problémů a nedostatků spojených s používáním větších zvířat a rovněž zdolat potíže s genetickou reprodukovatelností. Je vhodný také díky možnosti relativně velkého počtu experimentálních jedinců ve studiích zaměřených na léčbu. V současnosti jde o nejpoužívanější, nejekonomičtější a nejvhodnější model pro studium aterosklerózy a objasnění efektivního způsobu její léčby (Zadelaar 2007).

Po mnoho let se myší modely při studiu aterosklerózy nepoužívaly. Vědci se domnívali, že u myší se spontánně léze netvoří, že nejsou schopny přežívat na vysoce aterogenní tukové dietě, léze nejsou reprodukovatelné, a také že jejich patologie není podobná lidské. Nakonec se problém přežívání vyřešil použitím diety s nižším obsahem tuků, problém reprodukovatelnosti byl vyřešen využitím inbredních linií namísto náhodně vybíraných, ke snadné tvorbě aterosklerotických lézí došlo použitím tzv. rodově zatížených myších kmenů a tvorbu fibromuskulární čepičky umožnilo prodloužení experimentálního času (Shih 1995).

Je důležité si uvědomit hlavní rozdíly mezi myším a lidským organismem. Průměrná délka lidského života je okolo 75 let, zatímco u myši jsou to pouze 2 roky. Hmotnost myši je samozřejmě mnohem menší, v dospělosti kolem 30 gramů. Další rozdíl je v lipidovém spektru, které je velice odlišné od lidského. U člověka je většina plazmatického cholesterolu (až 75 %) obsažena v LDL, myši nemají

plazmatický cholesteryl ester transfer protein (CETP) (Moghadasian 2001) a většima cholesterolu je ve formě HDL (Jawien 2004). HDL cholesterol, jak je známo, představuje v lidském organismu ochranný faktor před vznikem aterosklerózy. Z tohoto důvodu u myší krmených normální nízkotučnou dietou se nevyvíjí ateroskleróza. Výhodou myších modelů, stejně tak jako všech ostatních zvířecích modelů, je možnost měnit vnější podmínky a dietu. U člověka to vzhledem k délce jeho života není možné. Při studiích s myšími modely jsou navíc možné různé genetické experimenty, zahrnující křížení a genetické inženýrství (Jawien 2004).

Využití myších modelů ve studiu aterosklerózy přináší řadu dalších výhod. Snadné a hospodárné je zejména jejich získávání a udržování druhu. Jejich reprodukční období je krátké (přibližně 9 týdnů - 3 týdny trvá období březosti a přibližně 6 týdnů dozrání do pohlavní dospělosti), proto je snadné odchovat velké skupiny pro experimentální studie. Klasická genetika je v myším organismu obzvlášť stabilní a tento fakt je ještě podpořen možností získat stovky inbredních linií (Jawien 2004).

Naopak hlavní nevýhodou myších modelů je jejich malá velikost, způsobující potíže při výkonu chirurgických manipulací a zobrazování *in vivo*.

Jak je uvedeno výše, myši jsou vysoce rezistentní vůči vzniku aterosklerózy a to díky vysoké hladině antiaterogenního HDL cholesterolu a nízkým hladinám proaterogenního LDL a VLDL cholesterolu. Výjimku však představuje **myší kmen C57BL/6J**. Aplikace diety bohaté na cholesterol s obsahem kyseliny cholové, vede u tohoto typu myší k tvorbě aterosklerotických lézí, které se ale od lidských odlišují v histologické povaze i umístění. Navíc jsou pravděpodobně způsobené spíše chronickým zánětem než genetickou predispozicí.

První použitá dieta vedoucí k rozvoji aterosklerózy u myši C57BL/6J obsahovala 30 % tuků, 5 % cholesterolu a 2 % žlučových kyselin. Bohužel se ukázalo, že je poměrně „drastická“, myši ubývaly na váze a často onemocněly smrtelnými respiračními infekcemi.

Další dietou byla tzv. „Paigenova dieta“, která obsahovala 15 % tuků, 1,25 % cholesterolu a 0,5 % kyseliny cholové (Paigen 1987). I když se tato dieta často používala, nebyla ideální. Myši byly krmené po dobu 14 týdnů až 9 měsíců. Aterosklerotické léze, které se u nich posléze vyvinuly, byly poměrně malé (200 -

1000 μm^2), byly omezeny převážně na aortální oblouk a nacházely se ve stádiu pěnových buněk s malým zastoupením hladkých svalových buněk. Na rozdíl od lidských lézí, zůstávaly v tomto případě léze ve stadiu tukových proužků a nedocházelo k jejich další progresi. Tato dieta rovněž není fyziologická s ohledem na extrémně vysoký podíl cholesterolu a přítomnost kyseliny cholové. Navíc bylo zjištěno, že tato dieta je sama o sobě prozánětlivá, jelikož vede k indukci jaterního NF-κB a expresi mediátorů akutní fáze zánětu, jako například sérového amyloidu A (Nishina 1993).

V roce 2002 zveřejnila Společnost pro sekvenování myšího genomu (The Mouse Genome Sequencing Consortium) vysoce kvalitní sekvenci a analýzu genomu myšího kmenu C57BL/6J (Waterston 2002). S nástupem molekulární genetiky je nyní možné začlenit do myšího genomu vnější geny, to je možné i u mnoha jiných živočišních druhů. Ale speciálně u myši, je rovněž možné vyřadit z funkce (knokautovat) nebo přemístit vnitřní geny, což je jedna z hlavních výhod práce s myšími modely. Všechny současně užívané myší modely pro studium aterosklerózy jsou založené na porušení lipoproteinového metabolismu spojením aterogenní diety a genetických manipulací (Jawien 2004).

Mezi nejvíce užívané myší modely pro studium aterosklerózy patří apolipoprotein E-deficientní (ApoE$^{-/-}$), LDL receptor-deficientní (LDLr$^{-/-}$) a ApoE/LDL receptor-deficientní myši. A novým modelem jsou ApoE*3Leiden (E3L) transgenní myši.

5.1.1. ApoE–deficientní myši (ApoE$^{-/-}$)

Jde o homozygotní myší kmen C57BL/6J bez genu pro apolipoprotein E (ApoE$^{-/-}$) zavedený v roce 1992. **Apo E** je glykoprotein syntetizovaný v játrech, v mozku a dalších tkáních, který má několik antiaterogenních funkcí. Je součástí lipoproteinových částic a slouží jako ligand pro buněčné receptory jako je LDL-receptor a receptor pro chylomikronové zbytky, čímž podporuje vychytávání aterogenních částic z oběhu. Deficit v apoE vede ke zpomalení clearance lipoproteinů. Tyto myši vyvíjejí spontánní hypercholesterolemii po podávání standardní diety, přičemž hladiny cholesterolu jsou 4-5 vyšší než u normálního kmene (Zhang 1992), zvyšuje se hladina celkového cholesterolu, dochází k nárůstu hladin zejména VLDL částic, chylomikronových zbytků a IDL částic. Po podání diety

s obsahem cholesterolu dochází ještě k výraznějšímu nárůstu hladin těchto částic (Ishibashi 1994). Tento model vyvíjí aterosklerotické léze všech fází s morfologickými charakteristikami blízce podobnými člověku a na stejných místech cévního stromu. Již kolem 4. až 5. týdne se na místech predisponovaných k ateroskleróze zvyšuje exprese adhezních molekul. Léze typu tukových proužků se začínají objevovat kolem 6. až 8. týdne věku a pokročilejší léze se objevují kolem 15. týdne života především v aortálním oblouku, karotidách a odstupech z aorty. Proces rozvoje aterosklerózy může být urychlen podáním vysokotukové nebo vysokocholesterolové diety (Moghadasian 2001).

Kromě toho se objevuje řada důkazů o tom, že apoE apolipoprotein má i další antiaterogenní vlastnosti. Uvažuje se například o tom, že apoE lipoprotein má také antioxidační, antiproliferativní, protizánětlivé, antiagregační vlastnosti, což souvisí s jeho potenciací sekrece NO (Ali 2005, Davignon 2005, Grainger 2004).

5.1.2. LDL receptor–deficientní myši (LDLr$^{-/-}$)

Další geneticky modifikovaný kmen je model **LDL receptor–deficientních myší** zavedený v roce 1993. Jde o model familiární hypercholesterolemie (Ishibashi 1993). Zastoupení lipoproteinových částic je u LDL receptor-deficientních myší podobné jako u člověka, tzn. většina cholesterolu je ve formě LDL a VLDL frakce. U myší, kterým chybí gen pro LDL receptor se objevuje po podání standardní diety mírně zvýšená hladina cholesterolu, a to zejména ve formě LDL částic a ateroskleróza se rozvíjí pozvolna. Avšak po podání diety s obsahem cholesterolu a tuku dojde k velkému nárůstu hladiny cholesterolu a k tvorbě detekovatelných lézí podobných jako u apoE-deficientních myší (Veniant 1998). Po podání diety s obsahem cholesterolu dochází k výraznému hromadění velkých lipoproteinových částic – chylomikronových zbytků, VLDL a IDL částic (Ishibashi 1994).

5.1.3. ApoE/LDL receptor-deficientní myši (ApoE$^{-/-}$/ LDLr$^{-/-}$)

Poslední skupinou myší o kterých bych se zmínila, a které byly vytvořeny genetickou manipulací, jsou tzv. dvojnásobně knokautované myši **apoE/LDL receptor-deficientní myši**. Tyto myši reprezentují zajímavý model, u kterého se vyskytuje kombinovaný defekt apoE lipoproteinu a LDL receptoru. Tento model je

schopen rozvinout závažnou hyperlipidémii a aterosklerózu. Bylo zjištěno, že u apoE/LDL receptor-deficientních myších dokonce i běžná strava vede k výraznější progresi aterosklerózy než u myší, které mají poruchu jen v apoE. Lipoproteinové spektrum je u těchto myší podobné jako u apoE modelu, zvýšená hladina VLDL částic a chylomikronových zbytků, ale také navíc LDL. Po podání diety s obsahem cholesterolu, dochází ještě k vyšší akumulaci těchto částic (Ishibashi 1994). Aterosklerotické léze jsou pozorovatelné již po 15 týdnech na normální dietě (Witting 1999). Z toho důvodu je ApoE$^{-/-}$/ LDLr$^{-/-}$ myší model vhodný pro studium antiaterosklerotického účinku jednotlivých látek bez nutnosti krmit zvířata aterogenní dietou.

5.1.4. ApoE*3Leiden (E3L) transgenní myši

ApoE*3-Leiden mutace je vzácná mutace v lidském genu *APOE3,* je spojována s familiární dysbetalipoproteinémií u lidí. ApoE*3Leiden (E3L) transgenní myši byly vytvořené vložením lidského *APOE*3-Leiden* segmentu do C57Bl/6 myší. Kromě genu *APOE*3-Leiden* obsahuje tento segment také *APOC1 gen* a promoter, který reguluje expresi *APOE* a *APOC1 genů.* Clearence lipoproteinů nesoucích apoE protein je tedy narušena, i když méně výrazně než u ApoE-deficientních myší. Zavedení *APOC1 genu* může dále zvýšit hladinu lipidů a to snížením lipolýzy a vychytávání VLDL částic prostřednictvím receptoru pro LDL a LRP receptoru pro chylomikronové zbytky (Zadelaar 2007).

E3L myši vykazují po podání normální diety významně zvýšené hladiny plazmatického cholesterolu a triacylglycerolů. Podání diety obsahující tuk nebo cholesterol vede u těchto myší k silnému nárůstu hladin plazmatického cholesterolu a triglyceridů, přičemž je významný zejména nárůst VLDL a LDL lipoproteinové frakce (Groot 1996).

U E3L myší se po podání diety s vysokým obsahem cholesterolu vyvíjejí aterosklerotické léze se všemi charakteristikami jako u člověka, od tukových proužků k závažným lézím. Ateroskleróza se vyvíjí nejprve v oblasti aortálního oblouku a pak se rozšiřuje na celý arteriální strom (Lutgens 1999).

5.2. Myší modely aterosklerózy a statiny

Statiny, kompetitivní inhibitory 3hydroxy-3-methyl-glutaryl-koenzym A-reduktázy, patří v současné době mezi nejúčinnější a celosvětově nejpoužívanější hypolipidemika. Přestože je prokázáno, že úspěšně snižují hladiny cholesterolu a také počet úmrtí z kardiovaskulárních příčin, stále se u 2/3 pacientů objevují závažné koronární příhody. V posledních letech dochází proto k rozmachu v oblasti vývoje látek, které by ovlivňovaly jiné rizikové faktory než hypercholesterolémii a daly by se používat v kombinaci se statiny, pro snížení rizika koronárních příhod (Zadelaar 2007)

Otázkou zůstává výběr vhodného zvířecího modelu, který by splňoval podmínky podobné těm v humánní medicíně.

U myších modelů je reakce na statiny velmi rozdílná (Zadelaar 2007).

U apoE-deficientních myší je účinek statinů na aterogenezi časově závislý. Při krátkodobém podávání statiny nesnižují hladinu plazmatického cholesterolu (Nachtigal 2006, Sparrow 2001). Zdálo se, že by mohly být vhodným modelem pro studium pleiotropních účinků statinů, ovšem při dlouhodobém podávání statinů dochází ke zvyšování hladiny cholesterolu (Fu 2006, Nachtigal 2006). Pozitivní vliv statinů byl sledován pouze u myší, které byly krmeny dietou s nízkým obsahem cholesterolu (Wang 2002) nebo u myší, kterým byly podávány statiny tzv. třetí generace – atorvastatin a rosuvastatin (Bisgaier 1997, Chen 2004). Ovšem přesto, že nedošlo k poklesu hladiny cholesterolu, některé statiny příznivě ovlivnily ukládání tuků ve stěně aorty. Při důkladném prozkoumání aterosklerotických lézí někteří autoři potvrzují antiaterogenní účinky – nižší výskyt krvácení uvnitř lézí, snížené ukládání vápníku (Bea 2003) a také zesílení fibrózní čepičky (Johnson 2005).

Podle dosavadních studií LDL receptor-deficientní myši reagují na podávání statinů variabilně. Nízké dávky pravastatinu neovlivnily významně aterosklerózu u těchto myší (Dunoyer-Geindre 2007, Kwak 2003) a to i přesto, že snížily hladinu plazmatického cholesterolu (Dunoyer-Geindre 2007). V dalších studiích byl používán simvastatin, který snížil velikost lézí a to jak v případě současného snížení hladiny plazmatického cholesterolu (Bea 2003), tak v případě, kdy hladina plazmatického cholesterolu zůstala stejná. (Chen 2002)

ApoE/LDLreceptor-deficientní myši reagují na podávání statinů příznivě. V našich pokusech podávání atorvastatinu vedlo ke snížení hladin všech lipoproteinových částic a navíc došlo k nárůstu hladiny HDL (Nachtigal 2008).

Podle dalších studií jsou vhodným modelem ApoE*3Leiden myši, jejichž reakce na statiny je podobná jako u lidí. Statiny mají hypolipidemické a antiaterosklerotické účinky (Delsing 2003, van Vlijmen 1998). Tento model je také vhodný pro studium pleiotropních účinků statinů (Kleemann 2003).

6. Cíle předkládané disertační práce

1. Studium exprese adhezních molekul a možnosti jejich ovlivnění po podávání statinů.
2. Studium exprese a lokalizace endoglinu v aortě.
3. Zavedení metody Western blot pro imunochemickou detekci adhezních molekul ve tkáních.

Podíl doktorandky na předkládaných publikacích:

U kapitoly IV. je předkladatelka této disertační práce první autorkou, v případě II, V, VI a VII je druhou autorkou a u kapitoly III pak spoluautorkou.

Autorka se podílela na přípravě diet, krmení a sledování zvířat, prováděla odběry a zpracování vzorků pro imunohistochemii, biochemii a Western blot. Stanovila podmínky pro stanovení exprese daných markerů ve tkáni metodou Western blot a podílela se na určení lokalizace exprese daných markerů imunohistochemicky.

Autorka disertace sepsala rukopis, u kterého je první autorkou. U dalších prací se podílela na sepisování zejména úvodních, metodických a výsledkových částí.

Stereologickou analýzu exprese daných markerů ve všech studiích prováděl PhamDr. Petr Nachtigal, PhD z Katedry biologických a lékařských věd Farmaceutické fakulty.

Biochemickou analýzu všech vzorků prováděla MUDr. Dagmar Solichová z Kliniky gerontologické a metabolické Fakultní nemocnice v Hradci Králové.

ELISA analýzu vzorků prováděl RNDr. Ctirad Andrýs, Ph.D. z Ústavu klinické imunologie a alergologie Lékařské fakulty v Hradci Králové.

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II.

MDOC™ AND ATORVASTATIN HAVE POTENTIAL ANTIINFLAMMATORY EFFECTS IN VASCULAR ENDOTHELIUM OF APOE(-/-) MOUSE MODEL OF ATHEROSCLEROSIS.

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MDOC™ and atorvastatin have potential antiinflammatory effects in vascular endothelium of apoE$^{-/-}$ mouse model of atherosclerosis

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Abstract

Members of the immunoglobulin superfamily of endothelial adhesion molecules, vascular cell adhesion molecule (VCAM-1) and intercellular cell adhesion molecule (ICAM- 1), strongly participate in leukocyte adhesion to the endothelium and play an important role in all stages of atherogenesis. The aim of this study was to detect and quantify the changes of endothelial expression of VCAM-1, and ICAM-1 in the vessel wall after the short-term administration of simvastatin, atorvastatin, and micro dispersed derivatives of oxidised cellulose (MDOC™) in apolipoprotein-E-deficient (apoE$^{-/-}$) mice atherosclerotic model. Hyperlipidemic apoE$^{-/-}$ mice (n=32) received normal chow diet or diet containing simvastatin or atorvastatin 10 mg/kg/day or MDOC™ 50 mg/kg/day. Total cholesterol, VLDL, LDL, HDL and TAG were measured and the endothelial expression of VCAM-1 and ICAM-1 was visualized and quantified by means of immunohistochemistry and stereology, respectively. Total cholesterol levels was insignificantly lowered only in MDOC™ treated mice but not in mice treated with statins. ICAM-1 endothelial expression was not affected by neither simvastatin nor MDOC™ treatment. However, significant diminution of VCAM-1 endothelial expression was observed in both atorvastatin and MDOC™ treated mice. These results provide new information of potential hypolipidemic substance MDOC™ and its potential anti-inflammatory effects. Furthermore, we have confirmed anti-inflammatory effects of atorvastatin independent of plasma cholesterol lowering. Thus, the results of this study show potential benefit of both MDOC™ and atorvastatin treatment in apoE$^{-/-}$ mouse model of atherosclerosis suggesting their possible combination might be of interest.

Keywords: Cell adhesion molecules; MDOC™; Atorvastatin; Simvastatin; Atherosclerosis; Mice

Introduction

The development of atherosclerotic lesions requires a complex interplay between mononuclear cells, endothelium, vascular smooth muscle, growth factors, and cytokines (Ross, 1999). Endothelial dysfunction followed by monocyte rolling and adhesion to the vascular endothelial lining and subsequent diapedesis are not only the first steps, but also seem to be crucial events in the atherosclerotic process (Nakashima et al., 1994). Several studies have demonstrated localized expression of leukocyte adhesion molecules in atherosclerotic lesions and plaques. They appear to regulate different stages of leukocyte migration at inflammatory sites in a multi-step process (Springer, 1994). Members of the immunoglobulin superfamily of endothelial adhesion molecules, vascular cell adhesion molecule (VCAM-1) and intercellular cell adhesion molecule (ICAM-1), strongly participate in leukocyte adhesion to the endothelium. VCAM-1 is highly expressed on endothelia prone to develop atherosclerosis in such atherosclerotic models as apoE$^{-/-}$ mice, LDL receptor- deficient mice (LDLR$^{-/-}$) mice, and rabbits fed with an atherogenic diet (Iiyama et al., 1999; Nakashima et al., 1998). ICAM-1 is expressed strongly on the endothelium overlying atheromatous plaque in human coronary

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and carotid arteries (DeGraba, 1997), hypercholesterolaemic rabbits (Iiyama et al., 1999), and apoE$^{-/-}$ (Nakashima et al., 1998) and LDLR$^{-/-}$ mice (Iiyama et al., 1999), although it is expressed in virtually all endothelial cells.

One of the most significant advances in drug therapy during the twentieth century was the development of the statin class of drugs. These agents inhibit the activity of 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMG-CoA) as well as induce up-regulation of LDL receptors on the cell surface (Vaughan et al., 1996). In addition, a growing body of evidence suggests that statins exert beneficial vascular effects that are independent of their cholesterollowering potencies (Farmer, 2000; LaRosa, 2001).

MDOC™ is micro dispersed derivatives of oxidized cellulose (polyanhydroglucuronic acid — PAGA). This semi-natural, biocompatible, bioabsorbable, non-acidic, sterile powder has been in use as a topical haemostatic agent since 1998. It is used to both stop bleeding and accelerate wound healing (Rysava et al., 2002). In addition, our preliminary experiment showed a strong hypolipidemic effect of MDOC™ in cholesterol-fed rabbits probably due to reduced absorption of cholesterol from the small intestine (unpublished data).

ApoE$^{-/-}$ mice, generated by gene targeting, have been shown to develop pronounced hypercholesterolemia and atherosclerotic lesions (Reddick et al., 1994) with certain features resembling those seen in humans (Nakashima et al., 1994) and other species (Davies et al., 1988). ApoE$^{-/-}$ mice exhibit spontaneous elevation of total plasma cholesterol and triglycerides and reduced levels of HDL on a diet with normal fat content and with no cholesterol supplementation (Zhang et al., 1992).

We hypothesized, that both statins and MDOC™ could have anti-inflammatory effects in the vessel wall in very early stages of atherogenesis regardless of their different mechanism of action on cholesterol metabolism. Thus, the aim of this study was to detect and quantify the changes of endothelial expression of VCAM-1, and ICAM-1 in the vessel wall after the shortterm administration of simvastatin, atorvastatin, and MDOC™ in apoE$^{-/-}$ mice atherosclerotic model.

Materials and methods

Animals

The Ethical Committee of the Faculty of Pharmacy, Charles University, approved the protocols of the animal experiments. The protocol of experiments was pursued in accordance with the directive of the Ministry of Education of the Czech Republic (No. 311/1997). Male apoE$^{-/-}$ mice on a C57BL/6J background (n=32) weighing 10–15 g were kindly provided by Prof. Poledne (IKEM, Prague, Czech Republic) and housed in the SEMED, (Prague, Czech Republic).

Experimental design

Male apoE$^{-/-}$ mice were weaned at 5 weeks of age and randomly subdivided into four groups. The control group of animals (n=8) was fed with the standard laboratory diet (chow diet) for another 4 weeks after the weaning. In both simvastatin (n=8) and atorvastatin (n=8) group, statins were added to the chow diet at the dosage of 10 mg/kg per day. In MDOC group (n=8) MDOC™ was added to the chow diet at the dosage 50 mg/kg per day. All treated mice were fed with the experimental diet for another 4 weeks after weaning with water ad libitum throughout the study. Each mouse, in both statins and MDOC group, lived in a separate cage obtaining 6 g of food (in specially prepared pellets) daily. The food consumption was monitored every day. No differences in the food consumption were visible, either among animals of one experimental group nor between experimental groups. The dose of simvastatin and atorvastatin used in the present study was based on the doses used in previous studies with hyperlipidemic mice (Laufs et al., 1998; Sparrow et al., 2001). The dose of MDOC™ was based on the results of a small pilot study.

At the end of the treatment period, all animals were fasted overnight and euthanized. Blood samples were collected via cardiac puncture at the time of death. The aortas, attached to the top half of the heart, were removed and then immersed in OCT (Optimal Cutting Temperature) embedding medium (Leica, Prague, Czech Republic), snap frozen in liquid nitrogen cooled 2-methylbutane and stored at −80 °C.

Biochemistry

Serum lipoprotein fractions were prepared using NaCl density gradient ultracentrifugation (Beckman TL 100, Palo Alto, CA). The lipoprotein fractions were distinguished in the following density ranges: very low density lipoprotein (VLDL)<1.006 g/ml; low density lipoprotein (LDL)<1.063 g/ml; high density lipoprotein (HDL)>1.063 g/ml. Total concentration and lipoprotein fraction concentration of cholesterol were assessed enzymatically by conventional diagnostic kits (Lachema, Brno, Czech Republic) and spectrophotometric analysis (cholesterol at 510 nm, triglycerides, at 540 nm wavelength), (ULTROSPECT III, Pharmacia LKB Biotechnology, Uppsala, Sweden).

Immunohistochemistry

Sequential tissue sectioning started in the mouse heart until the aortic root containing semilunar valves together with the aorta appeared. From this point on, serial cross-sections (7 μm) were cut on a cryostat and placed on gelatin-coated slides. Sections were air-dried and then slides were fixed for 20 min in acetone at −20 °C. Endogenous peroxidase activity was blocked with 0.3% hydrogen peroxide in phosphate buffered saline (PBS) for 15 min. After blocking of nonspecific binding sites with 10% normal horse serum (Sigma-Aldrich Chemie, Steinheim, Germany) in PBS solution (pH 7.4) for 30 min, slides were incubated with primary antibodies for 1 h at room temperature. After a PBS rinse, the slides were developed with biotinylated horse–anti goat IgG antibody or donkey anti-sheep IgG, in the presence of 200 mg/mL normal mouse IgG. Antibody reactivity was detected using HRPconjugated biotin–avidin complexes (Vector Laboratories, USA) and devel-

oped with diaminobenzidine tetrahydrochloride as substrate. Specificity of the immunostaining was assessed by staining with nonimmune isotype-matched immunoglobulins.

Primary antibodies included the following: polyclonal goat anti-mouse ICAM-1 (M- 19, IgG) and polyclonal goat anti-mouse VCAM-1 (C-19, IgG) diluted 1:100 purchased from Santa Cruz Biotechnology (California, USA) and sheep anti-human Von Willebrand factor (PC054 IgG) purchased from The Binding Site (Birmingham, England), diluted 1:300.

Quantitative analysis of the immunohistochemistry

Stereological methods for the estimation of immunohistochemical staining of VCAM-1, ICAM-1, and Von Willebrand factor were used as previously described (Nachtigal et al., 2002, 2004). In brief, the systematic uniform random sampling and the principle of the point-counting method were used for the estimation (Weibel, 1979). A total number of 50 consecutive serial cross-sections were cut into 7 ìm thick slices, which gave us 0.350 mm lengths of the vessel called the reference volume. This reference volume comprises several sections of the vessel containing semilunar valves in aortic root, and several sections of aortic arch (ascending part of the aorta). A systematic uniform random sampling was used in the reference volume. The first section for each immunohistochemical staining was randomly positioned in the reference volume and then each tenth section was used, thus five sections for each staining were used for the stereological estimation. The point-counting method was used and more than 200 test points per vessel, hitting immunostaining, were counted for an appropriate estimation (Gundersen et al., 1988). The estimated area is then:

$$\text{est}A = a^{*}P$$

where the parameter a characterizes the test grid and P is the number of test points hitting either the atherosclerotic lesion or positive immunostaining.

The area of Von Willebrand factor expression was considered as a total area of intact endothelium. Thus, the area of VCAM-1, and ICAM-1 expression indicates the percentage of activated endothelial cells calculated as

$$\text{est}P = \frac{\text{area}(x)}{\text{area}(\text{VonWill})}{}^{*}100\%,$$

where area (x) is the area of VCAM-1 or ICAM-1 in the endothelium and area (Von Will) is the area of Von Willebrand factor expression in the endothelium.

Photo documentation and image digitizing from the microscope were performed with the Nikon Eclipse E2000 microscope, with a digital firewire camera Pixelink PL-A642 (Vitana Corp. Ottawa, Canada) and with image analysis software LUCIA version 4.82 (Laboratory Imaging, Prague, Czech Republic). Stereological analysis was performed with a PointGrid module of the ELLIPSE software (ViDiTo, Kosice, Slovakia).

Statistical analysis

All values in the graphs are presented as a mean±SEM of $n=8$ animals. Statistical significance in the differences between groups was assessed by ANOVA followed by the Tukey test for multiple comparisons with the use of the SigmaStat software (version 3.0). P values of 0.05 or less were considered statistically significant.

Results

Biochemical analysis

Biochemical analysis showed that treatment with statins did not decrease levels of total cholesterol and VLDL (Fig. 1). Unlike statins, MDOC™ treatment resulted in a very mild and insignificant lowering of total serum cholesterol in comparison to the control group (17.35±2.64 vs. 21.62±2.94 mmol/l, $P=0.332$) (Fig. 1).

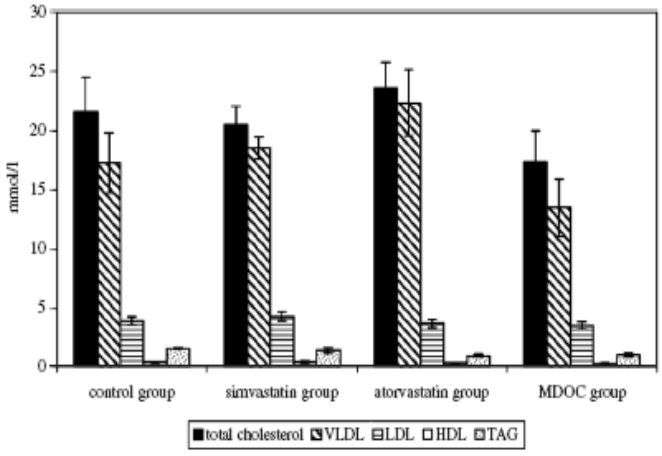

Fig. 1. Total serum cholesterol levels, VLDL, LDL, HDL and TAG in all mice. The results show that MDOC™ treatment slightly and insignificantly lowered cholesterol levels in comparison to the control group $p=0.332$. Statins did not affect lipid parameters in comparison to the control group.

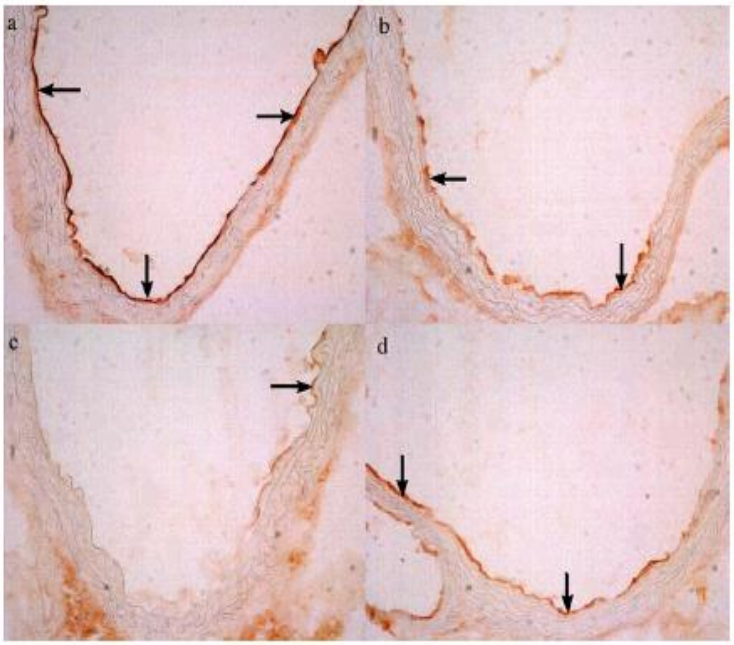

Fig. 2. The immunohistochemical staining of endothelial expression of ICAM-1 in all experimental animals. Note the strong endothelial expression in control animals (a) and weaker expression in simvastatin (b), and MDOC™ (d) treated animals. The ICAM-1 expression in atorvastatin treated animals (c) is visible in only a few endothelial cells (arrows). 200× original magnification.

Immunohistochemical staining of VCAM-1 and ICAM-1

No atherosclerotic lesion or other morphological abnormalities in either aortic root or aortic arch were visible in any mice in the experiment. Von Willebrand factor expression was observed only in endothelial cells in all groups of mice and this antibody was used as standard for the detection of intact endothelium (data not shown). The expression of VCAM-1 and

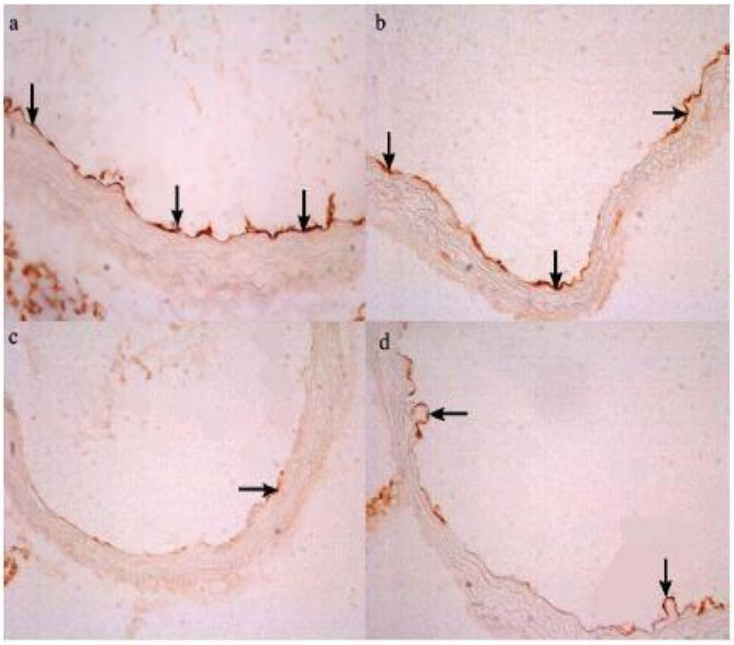

Fig. 3. The immunohistochemical staining of endothelial expression of VCAM-1 in all experimental animals. Note the strong endothelial expression in control animals (a) and slightly decreased expression in simvastatin (b) treated animals. Very weak endothelial expression of VCAM-1 (arrows) was detected in the atorvastatin (c) and MDOC group (d). 200× original magnification.

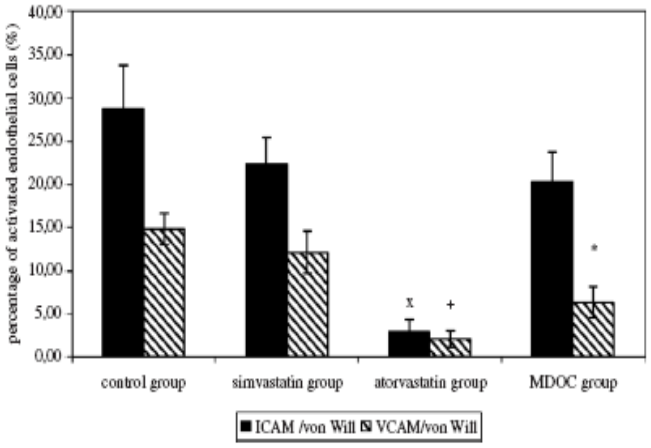

Fig. 4. The percentage of activated endothelial cells in both aortic root and aortic arch. The expression of VCAM-1 is significantly decreased in atorvastatin and MDOC™ treated mice in comparison to the control group. $^{*}P<0.001$ versus the control group. $^{+}P<0.001$ versus the control group. Moreover, atorvastatin significantly reduced expression of ICAM-1 in comparison to the control group. $^{x}P<0.001$ versus the control group.

ICAM-1 was observed in vessel endothelium in all groups of animals (Figs. 2 and 3). The ICAM-1 expression was stronger than VCAM-1 in each experimental group. Moreover, ICAM-1 expression decreased in both statins (Fig. 2b, c), and MDOC™ (Fig. 2d) treated mice compared to the control group (Fig. 2a). The same results were observed even for VCAM-1 staining (Fig. 3). However, the strongest diminution of ICAM-1 and VCAM-1 expression was visible in the atorvastatin treated mice (Figs. 2c and 3c).

Stereological analysis of ICAM-1 and VCAM-1 expression

The expression of ICAM-1 and VCAM-1 in endothelium was related to the von Willebrand staining of the endothelium, thus the results indicate the percentage of activated endothelial cells. Results of the stereological analysis confirmed that ICAM-1 staining was much stronger in all mice compared to the VCAM-1 staining (Fig. 4). The percentage of activated endothelial cells, ICAM-1/von Willebrand, was not changed in neither simvastatin (22.38±2.99 vs. 28.71±5.12%, P=0,259) nor MDOC™ (20.32±3.40 vs. 28.71±5.12%, P=0,171) treated animals in comparison to the control mice. However, significant diminution of both ICAM-1/von Willebrand (2.97±1.42 vs. 28.71±5.12%, $P<0.001$) and VCAM-1/von Willebrand (2.11±0.98 vs. 14.82±1.83%, $P<0.001$) staining was observed in atorvastatin treated animals in comparison to the control group (Fig. 4). In addition, VCAM-1/von Willebrand staining significantly decreased even in MDOC™ treated animals (3.35±1.80 vs. 14.82±1.83%, $P<0.001$) (Fig. 4).

Discussion

In the present study, we used the apoE$^{-/-}$ mouse to study potential anti-inflammatory effects of the short-term administration of simvastatin, atorvastatin, and MDOC™ in endothelium. We focused on the endothelial expression of ICAM-1 and VCAM-1 in the aortic root and aortic arch.

Clinical trials of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitor therapy demonstrate an improvement in cardiovascular end points and coronary stenosis (Schonbeck and Libby, 2004; Watanabe et al., 2004). Statins competitively inhibit HMG-CoA reductase, the enzyme that catalyzes the rate-limiting step in cholesterol biosynthesis. Additionally, the anti-inflammatory effects of statins were studied. It was found that statin treatment modulates inflammatory cytokines secreted by macrophages and T lymphocytes or endothelial cells including for instance monocyte chemotactic protein (MCP-1) (Ortego et al., 1999).

ApoE$^{-/-}$ mouse is a well-established genetic mouse model of atherogenic hypercholesterolemia, which is similar to hyperlipoproteinemia type III in humans. Moreover, it was demonstrated that the critical feature of this model is that statins do not decrease plasma lipid levels, and therefore the results may be interpreted without this confounding variable (Sparrow et al., 2001). In the present study, the short-term treatment with statins did not lower plasma total cholesterol as usually seen in humans (Ahmed and Griffiths, 2004). These results are consistent with the previous results described by Sparrow et al. (Sparrow et al., 2001), who showed that simvastatin treatment does not affect plasma cholesterol levels after six weeks dosing with 10 or 100mg/kg of simvastatin. On the contrary, other authors showed that treatment with statins results in paradoxical rise of plasma cholesterol in apoE$^{-/-}$ mouse suggesting that statins might reduce lipoprotein clearance (Bea et al., 2002; Wang et al., 2002). However, they dosed mice up to three months with the simvastatin and the initiation of treatment in these studies was different. Thus, it seems that initiation and duration of treatment with statins might be critical for their effect on the cholesterol level in apoE$^{-/-}$ mouse.

There is extensive evidence to suggest that monocytes play a pivotal role in the pathogenesis of atherosclerosis (Ross, 1999; Springer, 1994). Ligands for the two adhesion molecules examined in this study are expressed on the surfaces of monocytes and their expression is up-regulated upon monocyte

activation (Springer, 1995). Furthermore, many reports, including studies in mice, rabbits, and humans, concluded that the endothelial expression of several adhesion molecules, including ICAM-1 and VCAM-1, is increased at atherosclerosis prone sites and correlates with the extent of exposure to plasma cholesterol (Iiyama et al., 1999; Nakashima et al., 1998; Richardson et al., 1994). Thus, it can be argued that leukocyte and endothelial CAMs play a pivotal role in the pathogenesis of atherosclerosis, and that the effects of many risk factors might be mediated through effects on CAMs. In the present study, the immunohistochemical and stereological analysis showed that treatment with statins resulted in diminution of both ICAM-1 and especially VCAM-1 expression in endothelium. Sukhova et al. showed simvastatin to reduce the inflammatory reaction in atheroma of nonhuman primates independent of effects on serum cholesterol (Sukhova et al., 2002). Moreover, they described that VCAM-1 expression in the whole atherosclerotic lesion decreased in the monkeys treated with statins. During preparation of this manuscript, Li et al. demonstrated that rosuvastatin lowers plasma total cholesterol levels in apoE$^{-/-}$ mouse after 2 or 6 weeks of treatment (Li et al., 2005). This lipid lowering effect was accompanied by reduced expression of VCAM-1 and MCP-1. Thus, the present work confirms that statins are able to decrease the expression of VCAM-1 in the vessel wall. Moreover, it completes above-mentioned findings with the fact that atorvastatin is able to decrease the expression of both VCAM-1 and ICAM-1 only in endothelium before the formation of visible atherosclerotic lesion and beyond its cholesterol lowering effect.

MDOC™ is micro dispersed form of oxidised cellulose, which is formed by oxidation of cellulose in position C6 with following oxidative hydrolysis. (Patent Alltracel Pharmaceuticals, I.Santar et al. GB2335921). This process leads to the production of precisely specified highly pure biologically acceptable, and for the given purpose, well serving PAGA derivatives and/or highly pure PAGA itself. This may contain, in addition to C6OOH groups, other groups that lead to other possible derivatives with the ability to change confirmation of the chain according to the theory of hydrated surfaces, thus markedly influencing biological activity of these simply and cheaply prepared PAGA derivatives. It has been demonstrated that MDOC™ advances the immune system of an organism. In vivo MDOC™ application increases the number of colony forming units spleen (CFU-s) more than 50% i.e., stimulates stem cells in the bone marrow, and increases relative percentage of monocytes and B lymphocytes in the mouse peripheral blood (Jelinkova et al., 2002). Moreover, there have been numerous publications supporting a role for soluble fibres and gums in reducing serum cholesterol. These include psyllium and hydroxypropylmethylcellulose (Maki et al., 2000; Sierra et al., 2002). The mechanism mediating the hypocholesterolemic effect is incompletely understood. It is thought that the soluble fibres may form viscous gels in the intestine, which interfere with contact between the intestinal wall and the luminal contents. This has the effect of reducing or delaying the absorption of cholesterol (Carr et al., 1996). This theory may model MDOC™'s mechanism of action, but MDOC systems they have lower viscosity than other hydrocoloide systems (Levrat-Verny et al., 2000).

In contrast to the statins, we have demonstrated that the short-term administration of MDOC™ resulted in a very mild and insignificant hypocholesterolemic effect. This effect might be pronounced after longer treatment with MDOC™ because lipid metabolism shows a slower turnover and 4 weeks of lipid lowering treatment may not be sufficient. Despite this fact, stereological analysis of the immunohistochemical staining revealed that MDOC™ treatment resulted in significant decrease of VCAM-1 but no ICAM-1 endothelial expression. Thus, we suggest that this mild lipid lowering effect of MDOC™ might contribute to the diminution of VCAM-1 endothelial expression in early stages of atherogenesis. This suggestion is supported with the previous results by Aikawa et al. who demonstrated that VCAM-1 expression is decreased by dietary lipid lowering in rabbits (Aikawa et al., 2002).

The novel finding of this study is that the potential new hypolipidemic substance MDOC™ is able to decrease endothelial expression of VCAM-1 in very early stages of atherogenesis in apoE$^{-/-}$ mouse model of atherosclerosis. This potential anti-inflammatory effect might be related to the mild hypolipidemic effect of MDOC™. Moreover, we have demonstrated that atorvastatin reduces endothelial expression of both VCAM-1 and ICAM-1 independently of effects on serum cholesterol. Thus, this study confirms the acute antiinflammatory effects of atorvastatin and demonstrates potential anti-inflamatory effects of MDOC™. However, it must be stated that we have focused only on the initiation of the atherosclerotic process and on the expression of VCAM-1 in endothelium. It has been demonstrated that VCAM-1 is expressed even by macrophages and smooth muscle cells in the atherosclerotic lesions (Ley and Huo, 2001). Thus, other studies focusing on the expression of VCAM-1 and other inflammatory markers such as interleukin-6, MCP-1, or macrophage inhibitory factor (MIF) in more advanced atherosclerotic lesions must be made to prove antiinflammatory effects of MDOC™ and its possible combination with atorvastatin.

Conclusion

Our in vivo experimental results provide new information of potential hypolipidemic substance MDOC™ and its potential anti-inflammatory effects. Furthermore, we have confirmed that atorvastatin has acute anti-inflammatory effects independent of plasma cholesterol lowering. Thus, the results of this study show potential benefits of both MDOC™ and atorvastatin treatment in very early stages of atherogenesis in apoE$^{-/-}$ mouse model of atherosclerosis suggesting its possible combination might be of interest.

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III.

ATORVASTATIN HAS DISTINCT EFFECTS ON ENDOTHELIAL MARKERS IN DIFFERENT MOUSE MODELS OF ATHEROSCLEROSIS.

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Atorvastatin has distinct effects on endothelial markers in different mouse models of atherosclerosis

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ABSTRACT - Purpose. Atherosclerosis is a progressive process that initially involves endothelial dysfunction. We investigated the effects of atorvastatin on both lipid parameters, and VCAM-1 and ICAM-1 expression in apoE-deficient or wild type C57BL/6J mice. **Methods.** The C57BL/6J mice were fed with either chow or an atherogenic diet for 12 weeks. Male apoE-deficient mice were fed with the chow diet for 12 weeks. In 3 atorvastatin treated groups mice were fed the same diet as described above except atorvastatin was added to the diet at the dosage of 10 mg/kg per day for the last 8 weeks before euthanasia. **Results.** Biochemical analysis showed that atorvastatin significantly decreased total cholesterol levels and VLDL in C57BL/6J mice fed with atherogenic diet but increased serum lipid levels in apoE-deficient mice. Stereological analysis of the immunohistochemical staining revealed that atorvastatin reduced endothelial expression of ICAM-1 and VCAM-1 only in C57BL/6J mice on chow diet. **Conclusions.** We have demonstrated that endothelial expression of both VCAM-1 and ICAM-1 does not correlate with cholesterol levels in these mice. Moreover, we showed that 8-week administration of atorvastatin decrease endothelial expression of VCAM-1 and ICAM-1 in C57BL/6J wild type mice beyond its lipid lowering effect but not in C57BL/6J wild type mice fed by atherogenic diet or in apoE-deficient mice.

INTRODUCTION

Atherosclerosis is a progressive process that initially involves endothelial dysfunction and accumulation and peroxidation of intimal lipids, followed by release of inflammatory cells and growth factors, resulting in vascular smooth muscle cell (VSMC) proliferation and collagen matrix production [24]. Endothelial dysfunction followed by monocyte rolling and adhesion to the vascular endothelial lining and subsequent diapedesis are not only the first steps, but also seem to be crucial events in the atherosclerotic process [18]. Members of the immunoglobulin superfamily of endothelial adhesion molecules, vascular cell adhesion molecule (VCAM-1) and intercellular cell adhesion molecule (ICAM-1), strongly participate on leukocyte adhesion to the endothelium. VCAM-1 is highly expressed on endothelia prone to develop atherosclerosis in such atherosclerotic models as apoE-deficient mice ($apoE^{-/-}$), LDL receptor - deficient mice ($LDLR^{-/-}$), and rabbits fed with an atherogenic diet [12]. ICAM-1 is expressed strongly on the endothelium overlying atheromatous plaque in human coronary and carotid arteries [7], hypercholesterolemic rabbits [12], $apoE^{-/-}$ and $LDLR^{-/-}$ mice [19], although it is expressed in virtually all endothelial cells.

The advent of 3-hydroxy-3-methyl-glutaryl-coenzyme A (HMG-CoA) reductase inhibitors, or statins, has revolutionized the treatment of hypercholesterolemia. Statins competitively inhibit HMG-CoA reductase, the enzyme that catalyzes the rate-limiting step in cholesterol biosynthesis. In addition a growing body of evidence suggests that statins exert beneficial vascular effects that are independent of their cholesterol-lowering potencies [5].

It has been demonstrated that mice are highly resistant to atherosclerosis. However, when mice were fed by very high cholesterol, high-fat diet that also contained cholic acid, hypercholesterolemia is induced and atherosclerotic lesions are formed in the mouse aorta [20]. Moreover, other mouse models of atherosclerosis have been generated by gene knockout technology. ApoE-deficient ($apoE^{-/-}$) mice, have been shown to develop pronounced hypercholesterolemia and atherosclerotic lesions [23] on a diet with normal fat content and with no cholesterol supplementation [33]. It has been demonstrated that statins have diverse effects on atherogenesis in various mouse models of atherosclerosis [30].

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It has been shown that statins lower lipid levels in $LDLR^{-/-}$ mice [3], but not in $apoE^{-/-}$ mice [22] and wild type (WT) C57BL/6J mice [11]. Moreover, some conflicting data about statins effects on atherogenic changes in the vessel wall of apoE mice exist [25; 30].

Since above mentioned studies were made on older mice with advanced atherosclerotic lesions we focused only on the changes in the expression of some markers of endothelial dysfunction. We hypothesized whether atorvastatin affects the endothelial expression of VCAM-1 and ICAM-1 in $apoE^{-/-}$ and wild type C57BL/6J mice with different levels of cholesterol. Therefore, we investigated the effects of atorvastatin treatment on both lipid parameters, and on endothelial expression of VCAM-1 and ICAM-1 by means of immunohistochemistry and stereology.

The major findings of the present study are that atorvastatin has different effects on cholesterol levels and VCAM-1 and ICAM-1 endothelial expression in wild type C57BL/6J mice fed chow or atherogenic diet and $apoE^{-/-}$ mice.

MATERIALS AND METHODS

The Ethical Committee of the Faculty of Pharmacy, Charles University, approved the protocols of the animal experiments. The protocol of experiments was pursued in accordance with the directive of the Ministry of Education of the Czech Republic (No. 311/1997).

Experimental Animals

Male C57BL/6J mice and male homozygous apoE-deficient mice on a C57BL/6J background weighing 15-20 grams were kindly provided by Prof. Poledne (IKEM, Prague, Czech Republic) and housed in the SEMED (Prague, Czech Republic).

Experimental design

All mice were weaned at 4 weeks of age and randomly subdivided into six groups. The C57BL/6J mice (n=8) were fed with the chow diet or an atherogenic diet containing 1.25% cholesterol, 15% fat, and 0.5% cholic acid for 12 weeks after the weaning. Male apoE-deficient mice were fed with the chow diet for the same 12 weeks after the weaning. In three atorvastatin treated groups mice were fed the same diet as described above except atorvastatin was added to the diet at the dosage of 10 mg/kg per day for the last 8 weeks before euthanasia. Each mouse in atorvastatin treated groups lived in a separate cage obtaining 6 g of food (in especially prepared pellets) daily with water ad libitum throughout the study. The food consumption was monitored every day. No differences in the food consumption were visible neither among animals of one experimental group nor between experimental groups. The dose of atorvastatin used in the present study was based on the doses used in previous study with mice [8; 28].

At the end of the treatment period, all animals were fasted overnight and sacrificed. Blood samples were collected via cardiac puncture at the time of death. The aortas, attached to the top half of the heart, were removed and then immersed in OCT (Optimal Cutting Temperature) embedding medium (Leica, Prague, Czech Republic), snap frozen in liquid nitrogen cooled 2-methylbutane and stored at -80°C.

Biochemistry

Serum lipoprotein fractions were prepared using NaCl density gradient ultracentrifugation (Beckman TL 100, Palo Alto, CA). The lipoprotein fractions were distinguished in the following density ranges: very low density lipoprotein (VLDL) < 1.006 g/ml; low density lipoprotein (LDL) < 1.063 g/ml; high density lipoprotein (HDL) > 1.063 g/ml. Total concentration and lipoprotein fraction concentration of cholesterol were assessed enzymatically by conventional diagnostic kits (Lachema, Brno, Czech Republic) and spectrophotometric analysis (cholesterol at 510 nm, triglycerides, at 540 nm wavelength), (ULTROSPECT III, Pharmacia LKB Biotechnology, Uppsala, Sweden).

Immunohistochemistry

Sequential tissue sectioning started in the mouse heart until the aortic root containing semilunar valves together with the aorta appeared. From this point on, serial cross-sections (7μm) were cut on a cryostat and placed on gelatin-coated slides. Sections were air-dried and then slides were fixed for 20 minutes in acetone at -20°C. Endogenous peroxidase activity was blocked with 0.3% hydrogen peroxide in phosphate buffered saline (PBS) for 15 minutes. After blocking of nonspecific binding sites with 10% normal horse

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serum (Sigma-Aldrich Chemie, Steinheim, Germany) in PBS solution (pH 7.4) for 30 min, slides were incubated with primary antibodies for 1 hour at room temperature. After a PBS rinse, the slides were developed with biotinylated horse-anti goat IgG antibody or donkey anti-sheep IgG, in the presence of 200microg/mL normal mouse IgG. Antibody reactivity was detected using HRP (horse radish peroxidase)-conjugated biotin-avidin complexes (Vector Laboratories, USA) and developed with diaminobenzidine tetrahydrochloride as substrate. Specificity of the immunostaining was assessed by staining with nonimmune isotype-matched immunoglobulins.

Primary antibodies included the following: monoclonal antibody Rat Anti-Mouse CD31 (platelet endothelial cell adhesion molecule, PECAM-1) monoclonal antibody diluted 1:100, Rat Anti-Mouse CD106 (VCAM-1) diluted 1:100, and monoclonal antibody Hamster Anti-Mouse CD54 (ICAM-1) diluted 1:200. All antibodies were purchased from BD Pharmingen (California, USA)

Quantitative analysis of the immunohistochemistry

Stereological methods for the estimation of immunohistochemical staining of VCAM-1, ICAM-1, and PECAM-1 were used as previously described [16; 17]. In brief, the systematic uniform random sampling and the principle of the point-counting method were used for the estimation [32]. A total number of 50 consecutive serial cross sections were cut into 7μm thick slices, which gave us 0.350 mm lengths of the vessel called the reference volume. This reference volume comprises several sections of the vessel containing semilunar valves in aortic root, and several sections of aortic arch (ascending part of the aorta). A systematic uniform random sampling was used in the reference volume. The first section for each immunohistochemical staining was randomly positioned in the reference volume and then each tenth section was used, thus five sections for each staining were used for the stereological estimation. The point-counting method was used and more than 200 test points per vessel, hitting immunostaining, were counted for an appropriate estimation [9]. The estimated area is then:

$$estA = a * P$$

where the parameter a characterizes the test grid and P is the number of test points hitting either the atherosclerotic lesion or positive immunostaining.

The area of PECAM-1 expression was considered as a total area of intact endothelium. Thus, the area of VCAM-1, and ICAM-1 expression indicate the percentage of activated endothelial cells calculated as

$$estP = \frac{area(x)}{area(PECAM)} * 100\%,$$

where area (x) is the area of VCAM-1 or ICAM-1, in the endothelium and area (PECAM) is the area of PECAM-1 expression in the endothelium.

Photo documentation and image digitizing from the microscope were performed with the Nikon Eclipse E200 microscope, with a digital firewire camera Pixelink PL-A642 (Vitana Corp. Ottawa, Canada) and with image analysis software LUCIA version 5.0 (Laboratory Imaging, Prague, Czech Republic). Stereological analysis was performed with a PointGrid module of the ELLIPSE software (ViDiTo, Kosice, Slovakia).

Statistical analysis

All values in the graphs are presented as a mean ± SEM of n=8 animals. Statistical significance in the differences between groups was assessed by unpaired t-test with the use of the SigmaStat software (version 3.0). P values of 0.05 or less were considered statistically significant.

RESULTS

Biochemical analysis

Biochemical analysis showed that administration of atorvastatin to C57BL/6J mice fed with cholesterol and cholic acid resulted in significant decrease of total cholesterol levels (5.41 ± 0.35 vs. 4.11 ± 0.24 mmol/l, P = 0.005) and VLDL cholesterol levels (3.45 ± 0.38 vs. 1.99 ± 0.21 mmol/l, P = 0.002) in comparison to non-treated mice. Atorvastatin did not affect total cholesterol levels in WT C57BL/6J mice on chow diet (3.18 ± 0.36 vs. 2.69 ± 0.21 mmol/l, P = 0.277). Moreover, no effect of atorvastatin treatment on cholesterol fractions was detected in these mice as well. By contrast, in apoE-deficient mice, atorvastatin treatment significantly increased levels of total serum cholesterol (11.21 ± 0.75 vs. 17.51 ± 1.66 mmol/l, P = 0.005),

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VLDL cholesterol levels (8.49 ± 0.65 vs. 14.33 ± 0.89 mmol/l, P = <0.001), LDL cholesterol levels (2.46 ± 0.30 vs. 3.51 ± 0.30 mmol/l, P = 0.036), and so HDL cholesterol levels (0.23 ± 0.05 vs. 0.40 ± 0.04 mmol/l, P = 0.029) in comparison to the non-treated mice (Fig. 1).

Immunohistochemical staining of VCAM-1 and ICAM-1 in the endothelium

PECAM-1 expression was observed only in endothelial cells in all groups of mice and this antibody was used as standard for the detection of intact endothelium (data not shown). The expression of VCAM-1 and ICAM-1 was observed in vessel endothelium in all groups of animals (data not shown). Moreover the ICAM-1 expression was stronger then VCAM-1 in each experimental group. The expression of both VCAM-1 and ICAM-1 decreased only in C57BL/6J on chow diet treated with atorvastatin when compared to non-treated mice (Fig. 2 A-D). Atorvastatin treatment did not affect the expression of neither VCAM-1 nor ICAM-1 in both atherogenic diet fed animals and apoE-deficient mice (data not shown).

Stereological analysis of VCAM-1 and ICAM-1 immunohistochemical staining.

Quantitative stereological analysis of ICAM-1 and VCAM-1 endothelial expression was related to the PECAM-1 staining in endothelium, thus the results indicate the percentage of activated endothelial cells. It was demonstrated that PECAM-1 is not regulated by hypercholesterolemia thus it is used as marker of all (activated or non-activated endothelial cells) [19]. Atorvastatin treatment did not affect the expression of ICAM-1 in neither, mice fed by atherogenic diet (68.8 ± 5.6 vs. 68.4 ± 7.9%, P = 0.970) nor in apoE-deficient mice in comparison to non-treated mice (36.7 ± 5.1 vs. 49.4 ± 8.1%, P = 0.328) (Fig. 3). However, significant diminution of ICAM-1 (58.0 ± 3.6 vs. 43.8 ± 3.4%, P = 0.018) staining was observed in atorvastatin treated C57BL/6J mice on chow diet in comparison to the non-treated mice (Fig. 3). The results from stereological analysis of VCAM-1 staining were similar to the ICAM-1 expression. Atorvastatin significantly decreased VCAM-1 expression in C57BL/6J mice on chow diet only (37.3 ± 5.8 vs. 20.9 ± 6.4 %, P = 0.005) (Fig. 4). In contrast VCAM-1 staining was not affected by the atorvastatin treatment in neither mice fed by atherogenic diet (38.7 ± 6.6 vs. 41.0 ± 4.3%, P = 0.788) nor apoE-deficient mice (39.4 ± 5.1vs. 41.4 ± 6.6 %, P=0.959) (Fig. 4).

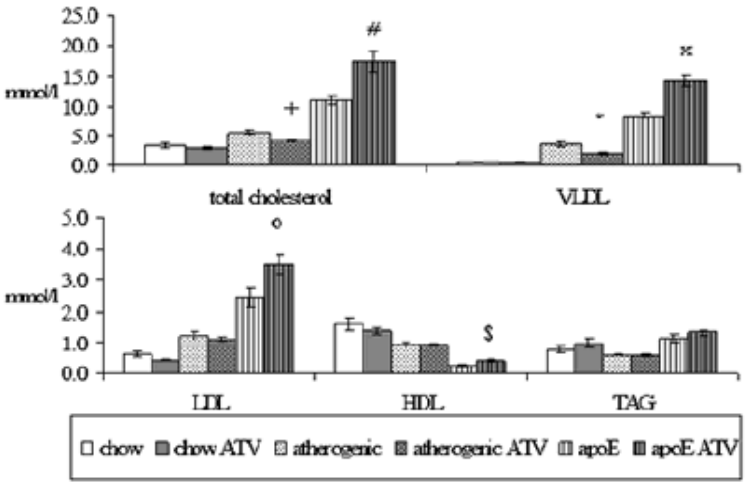

Figure 1: Serum lipid levels in all experimental mice. Atorvastatin significantly decreases total cholesterol levels ($^{+}$P = 0.005 versus non-treated mice) and VLDL cholesterol (*P = 0.002 versus non-treated mice) in C57BL/6J mice fed with atherogenic diet. On the contrary, atorvastatin significantly increases levels of the total cholesterol ($^{\#}$P = 0.005 versus non-treated mice), levels of VLDL cholesterol (^{x}P = <0.001 versus non-treated mice), levels of LDL cholesterol (^{o}P = 0.036 versus non-treated mice) and HDL cholesterol ($^{\$}$P = 0.029 versus non-treated mice) in apoE-deficient mice.

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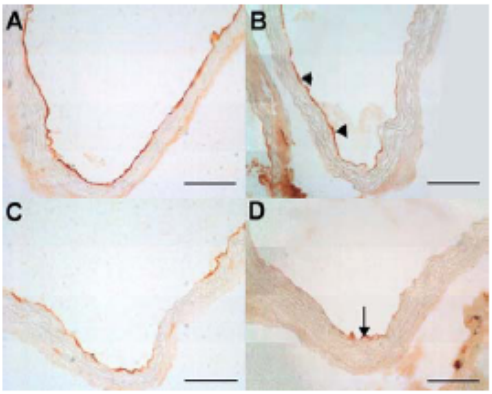

Figure 2: The immunohistochemical staining of endothelial expression of ICAM-1 (A, B), and VCAM-1 (C, D) in the aorta C57BL/6J mice on chow diet. Atorvastatin treatment notably reduced the expression of ICAM-1 (arrowhead) (B) when compared to non-treated C57BL/6J mice (A). VCAM-1 staining was markedly decreased (arrow) in atorvastatin treated animals (D). Bar = 50 µm

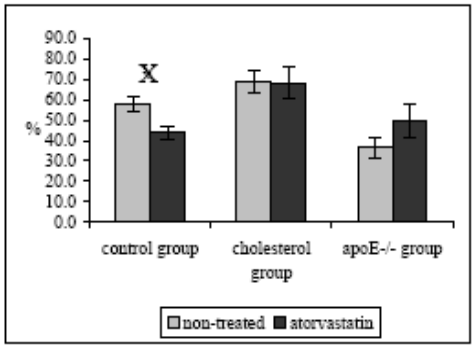

Figure 3: The percentage of activated endothelial cells for ICAM-1 in both aortic root and aortic arch. The expression of ICAM-1 was significantly decreased in C57BL/6J mice on chow diet after atorvastatin treatment (xP = 0.018 versus non-treated C57BL/6J mice). Atorvastatin treatment did not affect ICAM-1 staining in neither cholesterol fed animals, nor apoE-deficient mice.

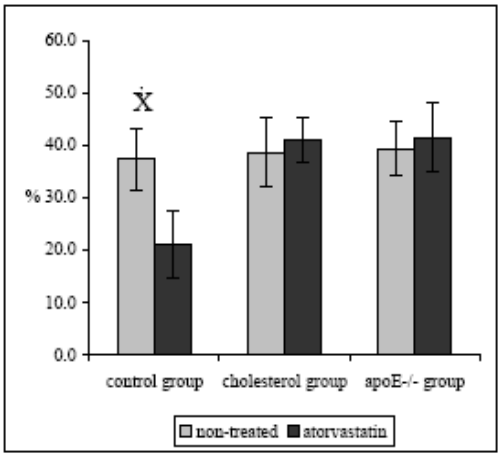

Figure 4: The percentage of activated endothelial cells for VCAM-1 in both aortic root and aortic arch. Atorvastatin treatment resulted in a significant diminution of endothelial expression of VCAM-1 in C57BL/6J mice on chow diet (xP = 0.005 versus non-treated C57BL/6J mice). VCAM-1 expression was not affected by the administration of atorvastatin in neither cholesterol fed mice, nor apoE-deficient mice.

DISCUSSION

A few years ago, several laboratories attempted to produce atherosclerosis in mice in order to identify potential modifier genes. Mice are highly resistant to atherosclerosis. On a low-cholesterol, low-fat diet, they typically have cholesterol levels of 2 mmol/l mostly contained in the antiatherogenic high density lipoprotein (HDL) fraction, and do not develop lesions. However, when mice were fed very high cholesterol, high-fat diet that also contained cholic acid, their cholesterol levels rose by a factor of two to three, with the majority now in the non-HDL fraction [4]. Moreover, genetic research and the application of transgenesis and gene targeting in mice resulted in generation of wide range of mice that are much more suitable atherosclerotic models including apoE-deficient mice, and $LDLR^{-/-}$ mice [10].

Clinical trials of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitor therapy demonstrate an improvement in cardiovascular end points and coronary stenosis [25; 31].

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However, it has been demonstrated the statins have various effects on cholesterol levels and atherogenic changes in the vessel wall in different mouse models of atherosclerosis [11; 30].

Thus, in this study we wanted to elucidate atorvastatin effects on both lipid parameters, and on endothelial expression of inflammatory markers VCAM-1 and ICAM-1 in WT C57BL/6J mice and apoE-deficient mice. The purpose was to reveal how atorvastatin affects the expression of VCAM-1 and ICAM-1 in these mice with different levels of cholesterol.

C57BL/6J mice are susceptible strain for the induction of atherosclerosis but diet saturated with fat, cholesterol and cholic acid must be used [21]. In our study, we found increased total serum cholesterol levels in group of mice after the feeding with diet containing 1.25% of cholesterol and 0.5% of cholic acid in comparision to mice fed by chow diet only. Several authors showed that prolonged feeding of this hyperlipidemic diet leads to the formation of atherosclerotic lesions which are restricted to the aortic root [20; 21]. However, we failed to detect any atherosclerotic lesions in neither cholesterol fed animals nor mice on chow diet in our study. This may be due to fact that period of feeding with atherogenic diet was not long enough in our study (12 weeks) in contrast to studies that used 14-18 weeks period of feeding [20].

ApoE- deficient mouse is a well-established genetic mouse model of atherogenic hypercholesterolemia, which is similar to hyperlipoproteinemia type III in humans. These mice lack their principal ligand for the LDL receptor and, therefore, develop hypercholesterolemia and atherosclerosis on diet with normal fat content [23]. We found the highest total serum cholesterol levels in apoE-deficient mice after 12 weeks on chow diet in comparison with C57BL/6J mice fed by either chow or atherogenic diet. Despite of this fact only small fatty streaks were visible in some mice in the aortic root.

Endothelial dysfunction followed by monocyte rolling and adhesion to the vascular endothelial lining and subsequent diapedesis are not only the first steps, but also seem to be crucial events in the atherosclerotic process [18]. Members of the immunoglobulin superfamily of endothelial adhesion molecules, vascular cell adhesion molecule (VCAM-1) and intercellular cell adhesion molecule (ICAM-1), strongly participate in leukocyte adhesion to the endothelium. The expression of VCAM-1 and ICAM-1 was largely studied by several authors in rabbit and mouse models of atherosclerosis [12; 13; 19]. It has been shown that VCAM-1 and ICAM-1 are detected in the regions predisposed to atherosclerotic lesion formation in normocholesterolemic rabbits, and the expression of both molecules is upregulated by a high-cholesterol diet in rabbits [12]. Furthermore, Nakashima described that VCAM-1 is upregulated in apoE -/- mice on Western diet when compared to animals on chow diet [19]. On the contrary, in our study we found that endothelial expression of both ICAM-1 and VCAM-1 is almost the same in non-treated C57BL/6J on chow diet, atherogenic diet and apoE-/- mice in spite of the different cholesterol levels. These results are consistent with Zibara et al. who showed no significant differences in VCAM-1 and ICAM-1 endothelial expression between 16 weeeks old apoE-/- mice on chow diet and C57BL/6J on chow diet [34]. Thus we suggest that endothelial expression of both VCAM-1 and ICAM-1 is not so strongly correlated by cholesterol levels in mice as it was demonstrated in rabbits [13; 19].

Statins competitively inhibit HMG-CoA reductase, the enzyme that catalyzes the rate-limiting step in cholesterol biosynthesis. The resultant reduction in hepatocyte cholesterol concentration triggers an increased expression of hepatic LDL receptors, which clear LDL and LDL precursors from the circulation [27]. Moreover, recent experimental and clinical evidence indicate that some of the cholesterol-independent or "pleiotropic" effects of statins involve improving or restoring endothelial function, enhancing the stability of atherosclerotic plaques, decreasing oxidative stress and inflammation, and inhibiting the thrombogenic response in the vascular wall [1].

In our study, atorvastatin treatment did not affect total cholesterol levels in C57BL/6J mice on chow diet which is in acceptance of Choudhury et al. who showed that simvastatin has no effect on cholesterol levels in C57BL/6J mice on chow diet [11]. However, we found statistically significant diminution of endothelial expression of VCAM-1 and ICAM-1 in aortic root and aortic arch of these mice. Thus, we suggest that lipid independent effects of atorvastatin are responsible for the reduction of VCAM-1 and ICAM-1 expression. The same effects of statins on cell adhesion molecules

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expression were described in monkeys [26] and apoE*-Leiden mice [29]

However we must emphasize that despite this positive influence of atorvastatin on endothelium, these mice on chow diet do not develop atherosclerosis, thus they cannot be used as model for the studying of statins in early atherogenesis. On the contrary one may speculate about their usage as model for the study of pleiotropic effects of statins in endothelium in normocholesterolemic conditions.

The administration of atorvastatin significantly decreased total cholesterol levels in mice fed with diet containing 1.25% cholesterol, 15% fat, and 0.5% cholic acid. However, despite this hypolipidemic effect of atorvastatin we failed to detect any changes in endothelial expression of VCAM-1 and ICAM-1. The explanation of this fact could be associated with potential inclusion of cholate in the diet. It has been demonstrated that administration of cholate in the diet lead to the induction of oxidative stress with activation of NF-κB transcription factor. Thus, cholate in the diet can cause inflammation to the vascular endothelium and initiate the atherogenic cascade [14]. Moreover it has been demonstrated that multiple genes whose products are putatively involved in the atherosclerotic process are regulated by NF-κB. This includes E-selectin, monocyte chemoattractant protein-1 (MCP-1) as well as VCAM-1 and ICAM-1 [6]. Thus, we suggest that despite the significant hypolipidemic effect of atorvastatin in cholesterol fed mice, potential proinflammatory effects cholic acid on endothelium could mask the benefit of atorvastatin treatment. Therefore we propose that this animal model is not suitable for the studying of statins effects in endothelium.

Atorvastatin treatment (8 weeks) resulted in a paradoxical elevation in plasma total cholesterol in our study in apoE-deficient mice. Other authors showed the same increase of total cholesterol in apoE-deficient mice suggesting that statins might reduce lipoprotein clearance [2; 30]. These hypercholesterolemic effects of statins were accompanied by accelerated atherosclerosis [2] suggesting that anti-atherosclerotic effects of statins depend on the presence of apolipoprotein E [30]. In agreement with these studies we found that 8 weeks atorvastatin treatment had no effect on the expression of VCAM-1 and ICAM-1, thus no benefit of atorvastatin treatment was observed. On the contrary in our previous work we showed that 4 weeks administration of atorvastatin reduced endothelial expression of both VCAM-1 and ICAM-1 beyond its lipid lowering [15]. In addition, under similar experimental conditions, Sparrow et al. [25], showed that simvastatin significantly decreased aortic cholesterol accumulation without significantly decreasing plasma cholesterol levels. These experimental data suggest that experimental design of the study, especially the age of animals, and the duration of statins treatment, might be critical for their effect on the cholesterol levels and atherogenesis in apoE-deficient mouse.

In conclusion, we have demonstrated that endothelial expression of both VCAM-1 and ICAM-1 is not strongly correlated with cholesterol levels in mice. Moreover, we showed that 8-week administration of atorvastatin decrease endothelial expression of VCAM-1 and ICAM-1 only in C57BL/6J wild type mice beyond its lipid lowering effect. However, we failed to detect this benefit of atorvastatin treatment on endothelium in C57BL/6J wild type mice fed by atherogenic diet or in apoE-deficient mice.

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IV.

ENDOGLIN EXPRESSION IN HYPER-CHOLESTEROLEMIA AND AFTER ATORVASTATIN TREATMENT IN APOE-DEFICIENT MICE

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Endoglin expression in hyper-cholesterolemia and after atorvastatin treatment in apoE-deficient mice.

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ABSTRACT – Purpose. Endoglin (CD105) is a marker of activated endothelium and a modulator of TGF-β signaling. We hypothesized whether endothelial expression of endoglin is changed in hypercholesterolemia as well as whether its expression is affected by atorvastatin treatment in apoE-deficient mice. **Methods.** ApoE-deficient mice were fed with the chow diet for either 4 weeks or for 12 weeks respectively. In two treated groups, mice were fed with chow diet except atorvastatin was added to the diet for the last 4 weeks or for the last 8 weeks respectively, before euthanasia. **Results.** Administration of atorvastatin did not affect lipid parameters after 4 weeks treatment, however increased all lipid parameters after 8 weeks of treatment. Stereological analysis of immunohistochemical staining revealed that atorvastatin significantly decreased endoglin expression in endothelium after 4 weeks of treatment but increased it after 8 weeks of treatment. **Conclusions.** This study demonstrate that endoglin is expressed by aortic endothelium showing similar staining patterns like other markers involved in the process of atherosclerosis. In addition, we showed that endoglin expression in endothelium could be affected by the administration of atorvastatin beyond its lipid lowering effects in apoE-deficient mice

INTRODUCTION

Atherosclerosis is a complex process that is characterized by the accumulation of modified low-density lipoprotein (LDL), local inflammatory and immune responses, and reduced nitric oxide bioavailability within the arterial wall (1). A central concept with regard to pathogenesis of atherosclerosis is that of endothelial cell dysfunction, which is associated with the release of a large number of mediators secreted predominantly by endothelial cells and leukocytes (2).

Transforming growth factor – beta (TGF-β), a widely expressed cytokine, is produced by both inflammatory and vascular cells and expressed in human and mouse atherosclerotic plaques (3). TGF-β exert their function through binding to a large family of specific receptors, including receptors type I, II, betaglycan, and endoglin (4). Among these, the serine-threonine kinase receptors types I and II are necessary for all tested biological responses to TGF-β and transmit the signal to downstream substrates through their kinase activity. By contrast, endoglin has been postulated as a regulator of TGF-β access to the signaling receptors (5).

Endoglin (CD105) is a homodimeric transmembrane glycoprotein composed of disulfide-linked subunits of 95 kDa. The primary sequence of human endoglin is composed of an extracellular domain of 561 amino acids, a single transmembrane region, and a cytoplasmic tail (6).

The major sources of CD 105 are vascular endothelial cells. Other cell types including vascular smooth muscle cells (7), fibroblasts (8), macrophages (9), leukemic cells of pre-B and myelomonocytic origin (10), and erythroid precursors (11) express CD105 to a lesser extent. Moreover endoglin is highly expressed in endothelial cells in tissues undergoing angiogenesis such as healing wounds, infarcts and in a wide range of tumors (12). In addition its expression was upregulated in human atherosclerotic plaques in the majority of smooth muscle cells (13).

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Lipid-lowering drugs offer one of the most effective therapeutic approaches used in clinical practice for the prevention and treatment of atherosclerosis (14). Statins, a well known class of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors, are active in the primary and secondary prevention of coronary heart disease and are the drugs most widely used for these purposes (15). A growing data base suggests that the beneficial actions of statins may be due to direct effects on the vascular wall in addition to lipid lowering. For instance, statins improve endothelial dysfunction via increased expression of nitric oxide (NO) and decreased expression of cell adhesion molecules in various animal models (16; 17).

Several papers provide evidence that TGF-β plays a major protective role in atherosclerosis (18). Moreover, as mentioned above, it has been demonstrated that endoglin is a modulator of TGF-β signaling and therefore it might affect these antiatherosclerotic effects of TGF-β. In addition, endoglin is a marker of activated endothelium (19; 20).

To the best to our knowledge nothing is known about the expression of endoglin in hypercholesterolemic conditions. Thus, in this study we wanted to evaluate the changes of endoglin expression in endothelium in very early stages of atherogenesis which comprise endothelial dysfunction. Moreover we hypothesized whether its expression is affected by an HMG Co-A reductase inhibitor atorvastatin. To test this hypothesis we examined the expression pattern of endoglin in endothelium in apoE-deficient mice by means of immunohistochemistry and stereology.

MATERIALS AND METHODS

The Ethical Committee of the Faculty of Pharmacy, Charles University, approved the protocols of the animal experiments. The protocol of experiments was pursued in accordance with the directive of the Ministry of Education of the Czech Republic (No. 311/1997).

Experimental Animals

Male homozygous apoE-deficient (apoE-/-) mice on a C57BL/6J background (n=32) weighing 15-20 grams were kindly provided by Prof. Poledne (IKEM, Prague, Czech Republic) and housed in the SEMED, (Prague, Czech Republic).

Experimental design

All mice were weaned at 4 weeks of age and randomly subdivided into four groups. Male apoE-deficient mice (n=8/group) were fed with the standard laboratory diet (chow diet) for either 4 weeks (non-treated apoE-/- 8 weeks) or for 12 weeks (non-treated apoE-/- 16 weeks) respectively, after the weaning. In the two atorvastatin treated groups, mice were fed with the standard laboratory diet, except atorvastatin was added to the diet at the dosage of 10 mg/kg per day for the last 4 weeks (ATV apoE-/- 8 weeks) or for the last 8 weeks (ATV apoE-/- 16 weeks) respectively, before euthanasia.

Each mouse in the atorvastatin groups lived in a separate cage obtaining 6g of food (in especially prepared pellets) daily with water ad libitum throughout the study. The food consumption was monitored every day. No differences in the food consumption were visible neither among animals of one experimental group nor between experimental groups. The dose of atorvastatin used in the present study was based on the doses used in previous studies with hyperlipidemic mice (21; 22).

At the end of the treatment period, all animals were fasted overnight and euthanized. Blood samples were collected via cardiac puncture at the time of death. The aortas, attached to the top half of the heart, were removed and then immersed in OCT (Optimal Cutting Temperature) embedding medium (Leica, Prague, Czech Republic), snap frozen in liquid nitrogen cooled 2-methylbutane and stored at -80°C until further analysis.

Biochemistry

Serum lipoprotein fractions were prepared using NaCl density gradient ultracentrifugation (Beckman TL 100, Palo Alto, CA). The lipoprotein fractions were distinguished in the following density ranges: very low density lipoprotein (VLDL) < 1.006 g/ml; low density lipoprotein (LDL) < 1.063 g/ml; high density lipoprotein (HDL) > 1.063 g/ml. Total concentration and lipoprotein fraction concentration of cholesterol were assessed enzymatically by conventional diagnostic kits (Lachema, Brno, Czech Republic) and spectrophotometric analysis (cholesterol at 510 nm, triglycerides, at 540 nm wavelength), (ULTROSPECT III, Pharmacia LKB Biotechnology, Uppsala, Sweden).

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Immunohistochemistry

Sequential tissue sectioning started in the mouse heart until the aortic root containing semilunar valves together with the aorta appeared. From this point on, serial cross-sections (7μm) were cut on a cryostat and placed on gelatin-coated slides. Sections were air-dried and then slides were fixed for 20 minutes in acetone at -20°C. Endogenous peroxidase activity was blocked with 3% hydrogen peroxide in phosphate buffered saline (PBS) for 15 minutes. After blocking of nonspecific binding sites with 10% normal goat serum (Sigma-Aldrich Chemie, Steinheim, Germany) in PBS solution (pH 7.4) for 30 min, slides were incubated with primary antibodies for 1 hour at room temperature. After a PBS rinse, the slides were developed with biotinylated goat-anti rat IgG antibody diluted 1:400 in the presence of 200 mg/mL normal mouse IgG. Antibody reactivity was detected using HRP-conjugated biotin-avidin complexes (Vector Laboratories, USA) and developed with diaminobenzidine tetrahydrochloride as substrate. Specificity of the immunostaining was assessed by staining with nonimmune isotype-matched immunoglobulins.

Primary antibodies included the following: monoclonal antibody Rat Anti-Mouse CD31 (PECAM-1) diluted 1:100, monoclonal antibody Rat Anti-Mouse CD105 (endoglin) diluted 1:50. All antibodies were purchased from BD Pharmingen (California, USA)

Quantitative analysis of the immunohistochemistry

Stereological methods for the estimation of immunohistochemical staining of endoglin, and PECAM-1 were used as previously described (23; 24). In brief, the systematic uniform random sampling and the principle of the point-counting method were used for the estimation (25). A total number of 50 consecutive serial cross sections were cut into 7μm thick slices, which gave us 0.350 mm lengths of the vessel called the reference volume. This reference volume comprises several sections of the vessel containing semilunar valves in aortic root, and several sections of aortic arch (ascending part of the aorta). A systematic uniform random sampling was used in the reference volume. The first section for each immunohistochemical staining was randomly positioned in the reference volume and then each tenth section was used, thus five sections for each staining were used for the stereological estimation. The point-counting method was used and more than 200 test points per vessel, hitting immunostaining, were counted for an appropriate estimation (26). The estimated area is then:

$$estA = a * P$$

where the parameter "a" characterizes the test grid and "P" is the number of test points hitting either the atherosclerotic lesion or positive immunostaining.

The area of PECAM-1 expression was considered as a total area of intact endothelium. Thus, the area of endoglin expression indicate the percentage of activated endothelial cells calculated as

$$estP = \frac{area(x)}{area(PECAM)} * 100\%,$$

where "area (x)" is the area of endoglin, in the endothelium and "area (PECAM)" is the area of PECAM-1 expression in the endothelium.

Photo documentation and image digitizing from the microscope were performed with the Nikon Eclipse E2000 microscope, with a digital firewire camera Pixelink PL-A642 (Vitana Corp. Ottawa, Canada) and with image analysis software LUCIA version 5.0 (Laboratory Imaging, Prague, Czech Republic). Stereological analysis was performed with a PointGrid module of the ELLIPSE software (ViDiTo, Kosice, Slovakia).

Statistical analysis

All values in the graphs are presented as a mean ± SEM of n=8 animals. Statistical significance in the differences between groups was assessed by unpaired t-test with the use of the SigmaStat software (version 3.0). P values of 0.05 or less were considered statistically significant.

RESULTS

Biochemical analysis

In the first place, we examined the changes in serum lipoprotein fractions in non-treated mice. Biochemical analysis surprisingly revealed that total cholesterol (21.62 ± 2.94 vs. 11.21 ± 0.75 mmol/l, P = 0,022), VLDL (17.28 ± 2.54 vs. 8.49 ± 0.65 mmol/l, P=0.003), LDL (3.92 ± 0.38 vs. 2.46 ± 0.30, P = 0.023), and HDL (0.42 ± 0.04 vs.

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0.23 ± 0.05, P = 0.043) levels were significantly decreased in 16 weeks old mice when compared with 8 weeks old mice (Fig.1).

Four weeks atorvastatin treatment significantly decreased TAG levels in 8 weeks old mice when compared to non-treated mice (1.57 ± 0.09 vs. 0.97 ± 0.12 mmol/l, P =0.014) (Fig.1). On the contrary, other lipid parameters were not affected by atorvastatin in these mice.

Eight weeks atorvastatin treatment significantly increased levels of the total serum cholesterol (11.21 ± 0.75 vs. 17.51 ± 1.16 mmol/l, P = 0.005), VLDL (8.49 ± 0.65 vs. 14.33 ± 0.89 mmol/l, P = <0.001), LDL (2.46 ± 0.30 vs. 3.51 ± 0.30 mmol/l, P = 0.036) and HDL (0.23 ± 0.05 vs. 0.40 ± 0.04 mmol/l, P = 0.029) in 16 weeks old mice in comparison to the non-treated mice (Fig.1).

Immunohistochemical staining of endoglin in apoE- deficient mice

ApoE-deficient mice develop spontaneous hypercholesterolemia on a chow diet which can be potentiated by atherogenic diet (27). However, the experimental design of this study was made to observe the changes of endoglin expression in endothelium where no atherosclerotic lesions were found. Thus no atherogenic diet was used.

PECAM-1 expression was observed only in endothelial cells in all groups of mice and this antibody was used as standard for the detection of intact endothelium (data not shown).

The staining patterns of endoglin were similar in both experimental groups. The expression of endoglin was detected in the endothelium of aorta in aortic sinus and aortic arch. Moreover, strong staining was visible in small vessel and capillaries in myocardium (data not shown).

Endoglin staining in aortic endothelium was lower in 8 weeks old mice treated with atorvastatin (Fig 2B) when compared with non-treated mice (Fig 2A). On the contrary atorvastatin treatment resulted in stronger expression of endoglin (Fig 2D) in 16 weeks old mice when compared with non-treated mice (Fig 2C).

Stereological analysis of endoglin expression in apoE-deficient mice

Quantitave stereological analysis of endoglin staining showed a significant decrease in its expression in non-treated 16 weeks old mice when compared with non-treated 8 weeks old mice (22.0 ± 4.5 vs. 6.6 ± 1.5 %, P=0.007) (Fig. 3).

Moreover we demonstrated a significant decrease of endoglin expression after 4 weeks administration of atorvastatin in comparison with non-treated 8 weeks old mice (22.0 ± 4.5 vs. 5.3 ± 1.2 %, P = 0.013) (Fig. 3).

By contrast 8 weeks atorvastatin treatment resulted in significant increase of the endoglin expression when compared to non-treated 16 weeks old mice (6.6 ± 1.5 vs. 23.5 ± 9.5 %, P = 0.021) (Fig. 3).

DISCUSSION

The novel findings of the present study is that endoglin is expressed by endothelium in aortic sinus and aortic arch in apoE-deficient mice, and moreover that its expression is affected by the atorvastatin treatment.

Endoglin, a homodimeric transmembrane glycoprotein, is a component of the TGF-β receptor complexes (28). The expression of endoglin is predominant in endothelial cells, macrophages, fibroblast, and medial SMCs (12). Moreover it has been demonstrated that endoglin expression is increased during angiogenesis, and tumor development (29). Furthermore, endoglin expression was upregulated in medial smooth muscle cells, and endothelial cells in advanced atherosclerotic lesions in porcine carotid artery (20).

Since nothing is known about endoglin expression in hypecholesterolemic conditions, we wanted to evaluate its staining patterns in very early stages of atherogenesis, namely endothelial dysfunction in apoE-deficient mouse model of atherosclerosis.

ApoE-deficient mouse is a well-established genetic mouse model of atherogenic hypercholesterolemia, which is similar to hyperlipoproteinemia type III in humans.

We found that total cholesterol, VLDL, LDL and HDL was significantly decreased in 16 weeks old mice when compared with 8 weeks old mice. We do not have explanation for this phenomenon. However this surprising decrease in cholesterol levels with age in apoE-deficient mice were demonstrated by other authors (30; 31).

Statins competitively inhibit HMG-CoA reductase, the enzyme that catalyzes the rate-limiting step in cholesterol biosynthesis.

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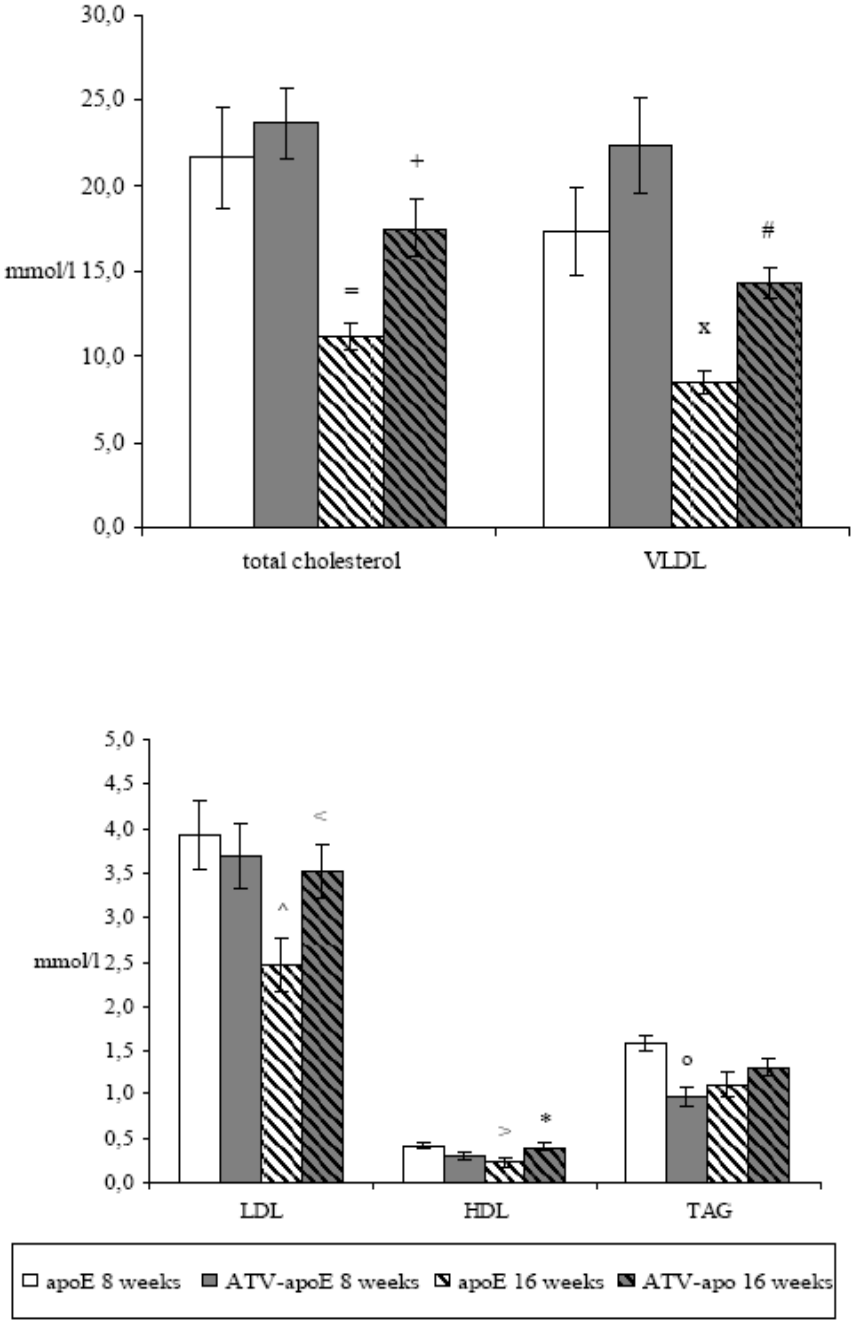

Figure 1. Serum lipid levels in apoE-deficient mice. Biochemical analysis revealed that in 16 weeks old non-treated mice there was significant decrease in levels of total cholesterol ($^{=}P = 0.022$), VLDL ($^{\#}P=0.003$), LDL ($^{\wedge}P = 0.023$) and HDL ($^{<}P = 0.043$) when compared with 8 weeks old non-treated mice. Four weeks atorvastatin treatment significantly decreases TAG levels in 8 weeks old mice ($^{\circ}P = 0.014$) when compared with non-treated mice. On the contrary in 16 weeks old mice 8 weeks atorvastatin treatment significantly increases levels of the total cholesterol ($^{+}P = 0.005$), VLDL ($^{\#}P = <0.001$), LDL ($^{>}P = 0.036$) and HDL ($^{*}P = 0.029$) when compared with non-treated mice. Male apoE-deficient mice (n=8/group) were used for the biochemical analysis.

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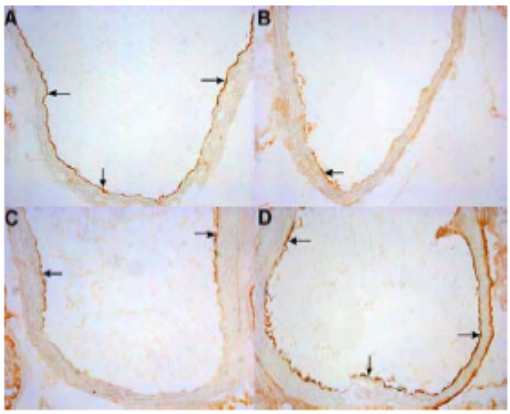

Figure 2. The figure demonstrates the intensity of immunohistochemical staining of endothelial expression of endoglin in non-treated and atorvastatin treated apoE-deficient mice. Endoglin staining (arrows) in aortic endothelium is lower in 8 weeks old mice treated with atorvastatin (B) when compared with non-treated mice (A). On the contrary atorvastatin treatment results in stronger expression of endoglin (arrows) (D) in 16 weeks old mice when compared with non-treated mice (C). Original magnification 200x.

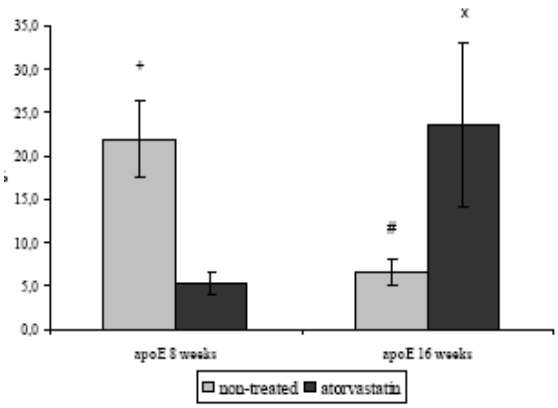

Figure 3. The percentage of activated endothelial cells for endoglin in both aortic root and aortic arch. In non-treated 16 weeks old mice the expression of endoglin is significantly decreased when compared with 8 weeks old mice ($^{\#}P=0.0073$). Endothelial expression of endoglin decreases after 4 weeks administration of atorvastatin in apoE-deficient mice ($^{+}P= 0.013$ versus non-treated group). By contrast 8 weeks atorvastatin treatment results in a significant increase of the endoglin expression ($^{x}P = 0.021$ versus non-treated mice. Male apoE-deficient mice (n=8/group) were used for the stereological analysis.

Moreover, recent experimental and clinical evidence indicate that some of the cholesterol-independent, or "pleiotropic" effects of statins involve improving or restoring endothelial function by enhancing the stability of atherosclerotic plaques, decreasing oxidative stress and inflammation, and inhibiting the thrombogenic response in the vascular wall (32).

We hypothesized, whether endothelial expression of endoglin is affected by atorvastatin treatment.

Endoglin expression was detected in myocardial capillaries and in endothelium of aortic sinus and aortic arch in all groups of mice showing similar staining pattern like cell adhesion molecules involved in the atherogenesis e.g. vascular cell adhesion molecule (VCAM-1) (33). Moreover, we demonstrated that 4 weeks administration of atorvastatin did not affect cholesterol levels in apoE-deficient mice, which is consistent with the results of Sparrow (22). However stereological analysis of immunohistochemical staining revealed that endothelial expression of endoglin was significantly lower in mice treated with atorvastatin. On the contrary 8 weeks atorvastatin treatment resulted in a paradoxical rise of all lipid parameters in apoE-deficient mouse. This effect of statin administration in apoE-deficient mice was previously described by other authors (30; 34; 35). This hypercholesterolemic effect of statins in apoE-deficient mice was recently elucidated by Fu et al., who demonstrated that enhanced hypercholesterolemia observed in apoE-deficient mice treated with statin is caused by alterations in the assembly of VLDL by the liver that contain more cholesterol than untreated mice (36). Moreover in our study, this hypercholesterolemic effect of atorvastatin was accompanied by the significant upregulation of endoglin expression in aortic endothelium in 16 weeks old mice. Thus, we suggest that hypercholesterolemic effect of atorvastatin overlay its direct effects on endothelium.

Taken together with the fact that decreased cholesterol levels in 16 weeks old non-treated mice was accompanied by the decrease in endoglin expression in endothelium when we compare it with 8 weeks old non-treated mice we might assume that the expression of endoglin could be affected by the changes of serum cholesterol in apoE-deficient mice. However, when we compare cholesterol levels and endoglin expression in 8 weeks and 16 weeks old

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atorvastatin treated mice we must state that this does not seem to be the case. Thus, cholesterol cannot be the only factor affecting the endothelial expression of endoglin. Therefore, we cannot exclude other factors e.g. proinflammatory state of endothelium that might be responsible for the increased endoglin expression in 16 weeks old atorvastatin treated mice.

As mentioned above, we showed that endoglin expression was reduced beyond lipid lowering effects of atorvastatin in 8 weeks old apoE-deficient mice.

It has been demonstrated that inhibition of HMG-CoA reductase by statins leads to the inhibition of nuclear transcription factor NF-kappaB which results in many beneficial pleiotropic effects of statins (37). Moreover, it has been demonstrated that multiple genes whose products are involved in the atherosclerotic process are regulated by NF-kappaB. This includes for instance E-selectin, monocyte chemoattractant protein-1 (MCP-1), and VCAM-1 (38). Furthermore, Rius et al. demonstrated that NF-kappaB consensus sequences found in the endoglin promoter might regulate endoglin transcription (39). Thus, it seems that the non-lipid lowering effect of atorvastatin on endoglin expression might be via an NF-kappaB dependent pathway. The same non-lipid lowering effect of atorvastatin on endothelial expression of VCAM-1 in apoE deficient mice was observed in our previous study suggesting both molecules might be regulated via an NF-kappaB dependent pathway (40)

TGF-β is a growth factor that exerts many regulatory actions. It is known for its role in development, proliferation, migration, differentiation, and extracellular matrix biology, but it is also an important immunomodulator (41). Moreover it has been demonstrated that endothelial cells and smooth muscle cells tend to be strongly inhibited by TGF-β both with respect to their proliferation and migration (42). In addition, macrophages and leukocytes are potently suppressed by TGF-β under most conditions tested (43). Thus, TGF-β is a strong anti-inflammatory agent that plays a protective role in the development of atherosclerosis (18). Since endoglin is a part of TGF-β receptor complex we might speculate that the expression of endoglin in the aorta could modulate the above mentioned TGF-β effects.

For instance it has been demonstrated that endoglin antagonizes the inhibitory effects of TGF-β and thus contributes to the proliferation, migration, and capillary formation of endothelial cells, the three key events in the angiogenic process (44). Moreover, endoglin is predominantly expressed in angiogenic endothelial cells and its expression is increased in tumor development. In addition endoglin expression was found to inhibit the TGF-β - dependent responses of cellular proliferation and PAI-1 expression (5).

If this inhibitory effect of endoglin would operate even for other effects of TGF-β, for instance inhibition of cell adhesion molecule expression (45) or vasodilatation, it is possible that increased expression of endoglin in hypercholesterolemia could results in inhibition of TGF-β signaling. In addition, this inhibition might result in the proinflammatory state in endothelium, increased activity of macrophages, T lymphocytes, thus actions that are necessary for the formation of atherosclerotic lesions.

In this study, we demonstrated that 4 weeks administration of atorvastatin decreases endothelial expression of endoglin. Thus, we assume that this decreased expression of endoglin by statin treatment might attenuate its inhibitory effects on TGF-β signaling which in turn could positively affect endothelial dysfunction in these mice.

On the other hand, increasing evidence indicates that endoglin may have functions independent of TGF-β. First, only a small percentage of surface-expressed endoglin actually binds TGF-β receptors (46). Second, despite the lack of signaling domains, endoglin overexpression affects cell morphology and adhesion in the absence of TGF-β (13). Thus, it is likely that endoglin cooperates with other different ligands. Thus, endoglin is a marker of activated endothelium regardless whether it affects TGF-β effects or not.

However, we must emphasize that this study was not designed to study possible interactions between TGF-β and endoglin. Therefore, further relation between endoglin and TGF-β in atherogenesis, as well as other effects of endoglin on markers of endothelial dysfunction for instance cell adhesion molecules expressions, such as NO production must be elucidated to reveal whether changes of endoglin expression could affect early atherogenesis.

There are some limitations of the study. First we used immunohistochemistry and stereology for the quantification of endoglin expression in endothelium in vessels where no atherosclerotic lesions were found. Thus, other

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studies focused on immunohistochemical and western blot analysis of endoglin expression in advanced atherosclerotic lesions must be made. Moreover, cell culture experiments with endothelial cells treated with or without atorvastatin in the presence or absence of cholesterol could confirm whether atorvastatin affects cholesterol-induced endoglin expression even in vitro.

In conclusion, this study demonstrate for the first time that endoglin is expressed by aortic endothelium showing similar staining patterns like other markers involved in the process of atherosclerosis. In addition, we showed that endoglin expression in endothelium could be affected by the administration of atorvastatin beyond its lipid lowering effects in apoE-deficient mice. Thus, prospective studies must be made to elucidate the role of endoglin and potential atorvastatin effects on its expression in more advanced atherosclerotic lesions and in relation to other markers of endothelial dysfunction and TGF-β signaling.

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V.

ENDOTHELIAL EXPRESSION OF ENDOGLIN IN NORMOCHOLESTEROLEMIC AND HYPERCHOLESTEROLEMIC C57BL/6J MICE AND AFTER ATORVASTATIN TREATMENT

Nachtigal P, **Pospisilova N,** Jamborova G, Pospechova K, Solichova D, Andrys C, Zdansky P, Semecky V: Endothelial expression of endoglin in normocholesterolemic and hypercholesterolemic C57BL/6J mice and after atorvastatin treatment. *Can. J. Physiol. Pharmacol.2007 Aug;85*(8):767-73

Endothelial expression of endoglin in normocholesterolemic and hypercholesterolemic C57BL/6J mice before and after atorvastatin treatment

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Abstract: Endoglin (CD105) is a homodimeric transmembrane glycoprotein strongly related to transforming growth factor (TGF)-β signaling and many pathological states. In this study, we wanted to evaluate whether endoglin is expressed in normocholesterolemic and hypercholesterolemic C57BL/6J mice as well as whether it is affected by atorvastatin treatment in these mice. C57BL/6J mice were fed with chow diet or an atherogenic diet for 12 weeks after weaning. In 2 atorvastatin-treated groups, mice were fed the same diets (chow or atherogenic) as described above except atorvastatin was added at the dosage of 10 $mg \cdot kg^{-1} \cdot day^{-1}$ for the last 8 weeks before euthanasia. Biochemical analysis of blood samples revealed that administration of atherogenic diet significantly increased levels of total cholesterol, VLDL, LDL, and decreased levels of HDL. Atorvastatin treatment resulted in a significant decrease in total cholesterol and VLDL only in mice fed by atherogenic diet. Quantitative stereological analysis revealed that atorvastatin significantly decreased endothelial expression of endoglin in C57BL/6J mice fed the atherogenic diet. In conclusion, we demonstrated that endothelial expression of endoglin is upregulated by hypercholesterolemia and decreased by the hypolipidemic effect of atorvastatin in C57BL/6J mice, suggesting that endoglin expression could be involved in atherogenesis.

Key words: endoglin, hypercholesterolemia, atorvastatin, C57BL/6J mice.

Résumé : L'endogline (CD105) est une glycoprotéine transmembranaire homodimérique étroitement associée à la voie de signalisation du TGF-β et à de nombreux états pathologiques. La présente étude a eu pour but d'évaluer si l'endogline est exprimée chez les souris C57BL/6J normocholestérolémiques et hypercholestérolémiques, et si elle est affectée par un traitement à l'atorvastatine. Les souris C57BL/6J ont été soumises à une diète normale de laboratoire ou à une diète athérogène pendant 12 semaines après le sevrage. Chez deux groupes traités à l'atorvastatine, les souris ont été soumises aux diètes (standard ou athérogène) décrites ci-dessus, auxquelles on a ajouté de l'atorvastatine, à raison de 10 $mg \cdot kg^{-1} \cdot jour^{-1}$ pendant les 8 dernières semaines respectivement, avant l'euthanasie. L'analyse biochimique des échantillons sanguins a révélé que le régime athérogène a augmenté significativement les taux de cholestérol total, de VLDL, et de LDL, et diminué les taux de HDL. Le traitement à l'atorvastatine a causé une diminution importante des taux de cholestérol total et de VLDL uniquement chez les souris ayant suivi le régime athérogène. L'analyse stéréologique a révélé que l'atorvastatine a diminué significativement l'expression endothéliale de l'endogline chez les souris C57BL/6J soumises au régime athérogène uniquement. Nous avons démontré que l'expression endothéliale de l'endogline est augmentée par l'hypercholestérolémie et diminuée par l'effet hypolipémiant de l'atorvastatine chez les souris C57BL/6J, ce qui laisse supposer que l'expression de l'endogline pourrait jouer un rôle dans l'athérogénèse.

Mots-clés : endogline, hypercholestérolémie, atorvastatine, souris C57BL/6J.

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Introduction

Endoglin (CD105) is a 190 kDa homodimeric transmembrane glycoprotein composed of 95 kDa disulfide-linked subunits (Guerrero-Esteo et al. 1999). The primary sequence of human endoglin is composed of an extracellular domain of 561 amino acids, a single transmembrane region, and a cytoplasmic tail (Zhang et al. 1996). Mutations in the gene encoding endoglin have been linked to the human disease hereditary hemorrhagic telangiectasia type 1 (HHT1), an autosomal dominant inherited vascular disorder (Li et al. 2000). The major sources of CD105 are vascular endothelial cells. Other cell types, including vascular smooth muscle cells (Adam et al. 1998), fibroblasts (St-Jacques et al. 1994), macrophages (Lastres et al. 1992), leukemic cells of preB and myelomonocytic origin (Kay et al. 2002), and erythroid precursors (Buhring et al. 1991), express CD105 to a lesser extent. Moreover, endoglin is highly expressed in endothelial cells in tissues undergoing angiogenesis, such as healing wounds, infarcts, and a wide range of tumors (Duff et al. 2003). In addition, its expression was upregulated in human atherosclerotic plaques in the majority of smooth muscle cells (Conley et al. 2000).

Lipid-lowering drugs offer one of the most effective therapeutic approaches used in clinical practice for the prevention and treatment of atherosclerosis (Arnaud and Mach 2005). Statins, a well-known class of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors, are active in the primary and secondary prevention of coronary heart disease and are the drugs most widely used for these purposes (Seljeflot et al. 2002). A growing database suggests that the beneficial actions of statins may be due to direct effects on the vascular wall in addition to their lipid-lowering abilities. For instance, statins improve endothelial dysfunction via increased expression of nitric oxide (NO) and decreased expression of cell adhesion molecules in various animal models (Cybulsky et al. 2001; Sukhova et al. 2002).

In our previous work, we found that endoglin is expressed by vascular aortic endothelium in apoE-deficient mice (Pospisilova et al. 2006). Moreover, we demonstrated that endoglin expression is decreased by the administration of atorvastatin beyond its lipid-lowering effects in these mice.

Thus, in this study, we wanted to expand our knowledge and evaluate whether endoglin is expressed in normocholesterolemic and hypercholesterolemic C57BL/6J mice, as well as whether it is affected by atorvastatin treatment in these mice. We focused on the immunohistochemical and stereological analysis of endoglin expression in endothelium of aortic sinus and aortic arch, but not in other tissues, because this is a reference for study of atherogenesis in mice and we believe that endoglin could be potential marker of early atherogenesis.

Materials and methods

Animals

The Ethical Committee of the Faculty of Pharmacy, Charles University, approved the protocols of the animal experiments. The protocol of experiments was pursued in accordance with the directive of the Ministry of Education of the Czech Republic (No. 311/1997).

Male C57BL/6J mice were obtained from Velaz (Velaz Ltd., Czech Republic).

Experimental design

All mice were weaned at 4 weeks of age and randomly subdivided into 4 groups. Male C57BL/6J mice (n = 8) were fed with a chow diet (standard laboratory diet) or an atherogenic diet containing 1.25% cholesterol, 15% fat, and 0.5% cholic acid for 12 weeks after the weaning. In 2 atorvastatin-treated groups, mice were fed with the same diets (chow or atherogenic diet) as described above except atorvastatin was added at the dosage of 10 $mg \cdot kg^{-1} \cdot day^{-1}$ for the last 8 weeks before euthanasia.

It is known that female C57BL/6J mice are more susceptible to the dietary-induced hypercholesterolemia and atherosclerosis when compared with male littermates (Paigen et al. 1987*a*). However, for the present study, we wanted to study the expression of endoglin only in endothelium without the presence of any atherosclerotic lesions; thus, we used male mice.

Each mouse in atorvastatin groups lived in a separate cage containing 6 g of food (in specially prepared pellets) daily with water ad libitum throughout the study. Food consumption was monitored every day. No differences in food consumption were visible among animals within an experimental group nor between experimental groups. The dose of atorvastatin used in the present study was based on the doses used in previous studies with hyperlipidemic mice (Laufs et al. 2000; Sparrow et al. 2001).

At the end of the treatment period, all animals were fasted overnight and euthanized. Blood samples were collected via cardiac puncture at the time of death. The aortas, attached to the top half of the heart, were removed and then immersed in OCT (optimal cutting temperature)-embedding medium (Leica, Prague, Czech Republic), snap frozen in liquid nitrogen-cooled 2-methylbutane, and stored at –80 °C.

Biochemistry

Serum lipoprotein fractions were prepared using sodium chlorate density gradient ultracentrifugation (Beckman TL 100, Palo Alto, Calif.). The lipoprotein fractions were distinguished in the following density ranges: very low density lipoprotein (VLDL) < 1.006 g/mL; low density lipoprotein (LDL) < 1.063 g/mL; high density lipoprotein (HDL) > 1.063 g/mL. Total concentration and lipoprotein fraction concentration of cholesterol were assessed enzymatically by conventional diagnostic kits (Lachema, Brno, Czech Republic) and spectrophotometric analysis (cholesterol at 510 nm, triglycerides at 540 nm wavelength, Ultrospect III, Pharmacia LKB Biotechnology, Uppsala, Sweden).

Immunohistochemistry

Sequential tissue sectioning started in the mouse heart until the aortic root, which contained semilunar valves together with the aorta, appeared. From this point on, serial cross sections (7 μm) were cut on a cryostat and placed on gelatin-coated slides. Sections were air dried and then slides were fixed for 20 min in acetone at –20 °C. After PBS (pH 7.4) rinse, slides were incubated with anti-avidin and antibiotin solutions (Vector Laboratories, USA) to decrease

negative background staining. After blocking of nonspecific binding sites with 10% normal goat serum (Sigma-Aldrich Chemie, Germany) in PBS solution (pH 7.4) for 30 min, slides were incubated with primary antibodies for 1 h at room temperature. After a PBS rinse, the slides were developed with biotin-conjugated goat anti-rat Ig (diluted 1/400 in BSA) (BD Pharmingen, Calif.) in the presence of 200 μg/mL normal mouse IgG (Dako, Denmark). Antibody reactivity was detected using HRP (horseradish peroxidase)-conjugated biotin–avidin complexes (Vector Laboratories, USA) and developed with diaminobenzidine tetrahydrochloride substrate (Dako, Denmark). Specificity of the immunostaining was assessed by staining with nonimmune isotype-matched immunoglobulins. Primary antibodies included the following: monoclonal antibody rat anti-mouse CD31 (PECAM-1) and monoclonal antibody rat anti-mouse CD105 (endoglin) diluted 1:50. All antibodies were purchased from BD Pharmingen (Calif.).

Quantitative analysis of the immunohistochemistry

Stereological methods for the estimation of immunohistochemical staining of endoglin and PECAM-1 were used as previously described (Nachtigal et al. 2002, 2004). In brief, the systematic uniform random sampling and the principle-of-the-point counting method were used for the estimation (Weibel 1979). A total of 50 consecutive serial cross sections were cut into slices 7 μm thick, which gave us 0.350 mm lengths of the vessel called the reference volume. This reference volume comprises several sections of the vessel containing semilunar valves in aortic root and several sections of aortic arch (ascending part of the aorta). A systematic uniform random sampling was used in the reference volume. The first section for each immunohistochemical staining was randomly positioned in the reference volume and then each tenth section was used. Thus 5 sections for each staining were used for the stereological estimation. The point-counting method was used and more than 200 test points per vessel that hit immunostained areas were counted for an appropriate estimation (Gundersen et al. 1988). The estimated area (A_{est}) is then:

$$A_{est} = a \times P$$

where the parameter a characterizes the test grid and P is the number of test points hitting either the atherosclerotic lesion or positive immunostaining.

The area of PECAM-1 (P_{est}) expression was considered as a total area of intact endothelium. Thus, the area of endoglin expression indicates the percentage of activated endothelial cells calculated as:

$$P_{est} = \frac{\text{area}(x)}{\text{area(PECAM)}} \times 100\%$$

where area (x) is the area of endoglin in the endothelium and area (PECAM) is the area of PECAM-1 expression in the endothelium.

Photo documentation and image digitizing from the microscope were performed with the Olympus AX 70, with a digital firewire camera Pixelink PL-A642 (Vitana Corp. Ottawa, Ont.) and with image analysis software NIS (Laboratory Imaging, Czech Republic). Stereological analysis was performed with a PointGrid module of the Ellipse software (ViDiTo, Slovakia).

Statistical analysis

All values in the graphs are presented as a mean ± SE of $n = 8$ animals. Statistical significance in the differences between groups was assessed by unpaired Student's t test with the use of the GraphPad Prism software (version 4.0). p values of 0.05 or less were considered statistically significant.

Results

Biochemical analysis

Biochemical analysis of blood samples revealed that administration of the atherogenic diet significantly increased levels of total cholesterol (3.18 ± 0.36 vs. 5.41 ± 0.35 mmol/L, $p = 0.0015$), VLDL (0.57 ± 0.13 vs. 3.45 ± 0.24 mmol/L, $p = 0.005$), and LDL (0.60 ± 0.08 vs. 1.20 ± 0.10 mmol/L, $p = 0.0028$) when compared with mice on chow diet. Moreover HDL levels were significantly decreased (1.61 ± 0.12 vs. 0.92 ± 0.08 mmol/L, $p = 0.0034$) after feeding with the atherogenic diet (Fig. 1).

Atorvastatin treatment did not affect any lipid parameters in C57BL/6J mice fed with chow diet (Fig. 1).

On the other hand, atorvastatin treatment significantly decreased total serum cholesterol (5.41 ± 0.35 vs. 4.11 ± 0.24 mmol/L, $p = 0.005$) and VLDL cholesterol (3.45 ± 0.38 vs. 1.99 ± 0.21 mmol/L, $p = 0.002$) in C57BL/6J mice fed with atherogenic diet in comparison with nontreated mice (Fig. 1). LDL and HDL cholesterol and triacylglycerol (TAG) levels were not affected by the treatment.

Immunohistochemical staining of endoglin in C57BL/6J mice

PECAM-1 expression was observed in endothelial cells of aorta, small vessels, and capillaries in surrounding myocardium in all groups of mice, and this antibody was used as the standard for the detection of intact endothelium (data not shown).

The staining patterns of endoglin were similar in all experimental groups. The expression of endoglin was detected in the endothelium of aorta in the aortic sinus and aortic arch (Fig. 2*a*). Moreover, strong staining was visible in small vessels and capillaries in myocardium. The main difference between experimental groups was in the intensity of endoglin staining in aortic endothelium, as shown.

Stereological analysis of endoglin expression in C57BL/6J mice

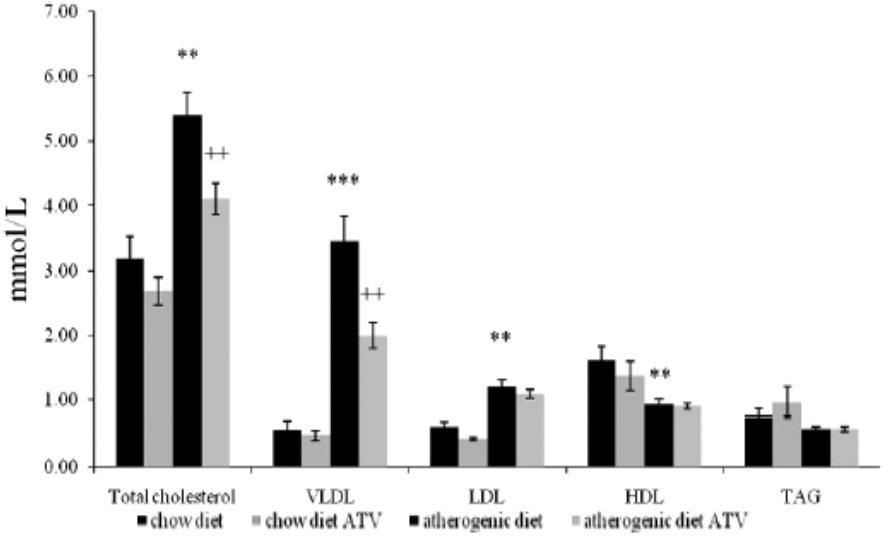
Quantitative stereological analysis of endothelial expression of endoglin was related to the PECAM-1 staining of the endothelium, thus the results indicate the percentage of activated endothelial cells.

Feeding with the atherogenic diet resulted in a significant increase in endothelial expression of endoglin (17.6% ± 5.0% vs. 37.1% ± 5.6%, $p = 0.036$) (Fig. 2*b*).

On the contrary, a significant decrease of endoglin expression was revealed in atorvastatin-treated C57BL/6J mice fed with atherogenic diet (37.1% ± 5.6% vs. 21.3% ± 4.8%, $p = 0.049$) (Fig. 2*b*).

However, atorvastatin treatment did not significantly af-

Fig. 1. Serum lipid levels in C57BL/6J mice fed by chow or atherogenic diets with or without atorvastatin (ATV) treatment. Atherogenic diet significantly increased total cholesterol, very low density lipoprotein (VLDL) and low density lipoprotein (LDL) levels when compared with mice fed with chow diet only. On the other hand, high density lipoprotein (HDL) levels were decreased after atherogenic diet. Atorvastatin treatment did not significantly affect any lipid parameters when compared with nontreated mice on chow diet, but significantly decreased total cholesterol and VLDL levels in mice fed with atherogenic diet. Other parameters were not affected by the treatment. Values are means ± SE, $n = 8$. Statistical significance at $^{**}p < 0.01$ and $^{***}p < 0.001$ vs. nontreated chow diet; $^{++}$, significant at $p < 0.01$ vs. nontreated atherogenic diet.



fect the expression of endoglin in C57BL/6J mice on the chow diet.

Discussion

Endoglin, a homodimeric transmembrane glycoprotein composed of 95 kDa disulfide-linked subunits, is a component of the transforming growth factor-β (TGF-β) receptor complexes (Bellon et al. 1993). The expression of endoglin is predominant in endothelial cells, macrophages, fibroblasts, and medial smooth muscle cells (Duff et al. 2003). The role of endoglin has largely been studied in various pathological states, including hereditary hemorrhagic teleangiectasia (van Laake et al. 2006; Llorca et al. 2007), cancer (El-Gohary et al. 2007; Nikiteas et al. 2007), and lately in preeclampsia (Levine et al. 2006; Lopez-Novoa 2007). Only a few studies have focused on the possible role of endoglin in atherogenesis. Conley et al. demonstrated that endoglin is expressed at low levels in normal porcine coronary arteries and, in addition, overexpressed at early intervals after balloon injury not only in endothelial cells and fibroblasts, but also transiently in smooth muscle cells and (myo)fibroblasts (Conley et al. 2000). Moreover, endoglin expression was undetectable in smooth muscle cells of the normal vessel wall; however, its expression was readily detected in smooth muscle cells within atherosclerotic plaques (Ma et al. 2000). These studies suggested that endoglin is required for smooth muscle development and that it participates in atherogenesis. Furthermore, Piao and Tokunada demonstrated endoglin expression in macrophages, endothelial cells, and smooth muscle cells in human atherosclerotic lesions (Piao and Tokunada 2006). In addition they described endoglin expression simultaneously with TGF-β1 and TGF-βR2 in atherosclerotic lesions but not in nonatherosclerotic aortas, suggesting that endoglin, or its receptor complex may participate in atherogenesis. However, to our knowledge, no one has proposed what causes increased levels of endoglin in atherosclerotic vessels. In our previous study, we showed that endoglin is expressed in endothelium of apoE-deficient mice (Pospisilova et al. 2006). Moreover, its expression was reduced by atorvastatin treatment beyond its lipid-lowering effects.

In this study, we used C57BL/6J mice to study endoglin expression in normocholesterolemic state after an atherogenic diet and after atorvastatin treatment. C57BL/6J mice represent an atherosclerosis-susceptible strain, but high cholesterol (atherogenic diet) is necessary for the induction of hypercholesterolemia, which is accompanied, however, only by the formation of very small lesions called fatty streaks (Breslow 1996). In our study, we demonstrated that administration of a high cholesterol diet containing 1.25% cholesterol, 15% fat, and 0.5% cholic acid for 12 weeks resulted in a significant increase of total cholesterol, VLDL, LDL, and a simultaneously significant decrease of HDL cholesterol when compared with control mice. These results are consistent with previous studies (Paigen et al. 1987*b*; Nishina et al. 1993). In addition, this hypercholesterolemic effect was accompanied by the significant increase of endothelial expression of endoglin in aortic sinus and aortic arch.

Statins competitively inhibit 3-hydroxy-3-methyl-glutaryl (HMG)-CoA reductase, the enzyme that catalyzes the rate-limiting step in cholesterol biosynthesis. Thus its lipid-lowering effect was demonstrated in both humans and other animals (Subang et al. 1992; Shiomi and Ito 1999; Rezaie-Majd et al. 2003; Wierzbicki et al. 2003).

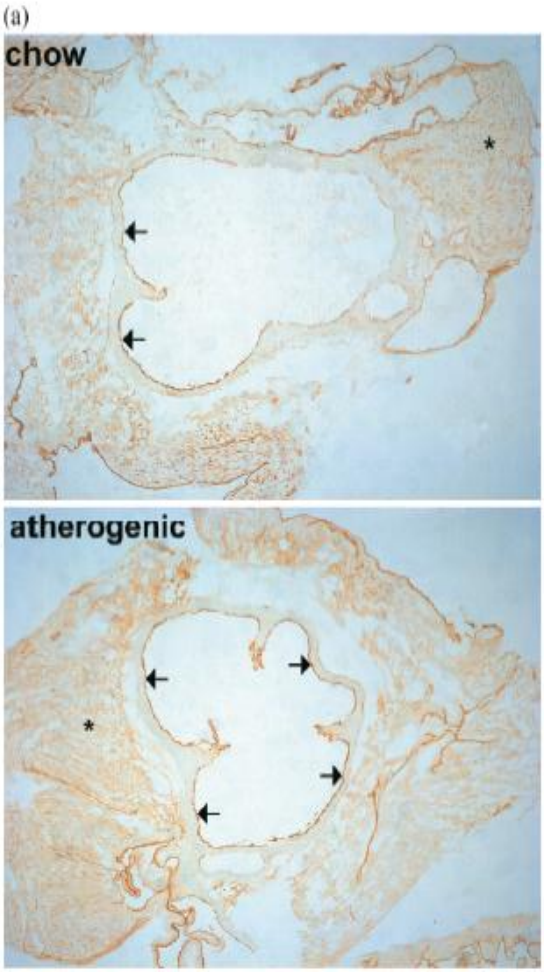


Fig. 2. (*a*) The immunohistochemical staining of endothelial expression of endoglin in C57BL/6J mice on chow or atherogenic diets. Similar staining patterns were visible in all mice in the experiment. The expression of endoglin was detected in endothelium of aorta in the aortic sinus and arch (arrows). Moreover, strong staining was visible in capillaries in surrounding myocardium (asterisk) as well. Note the stronger intensity of endoglin staining in aortic endothelium in mice on atherogenic diet compared with mice on chow diet. Original magnification ×40. (*b*) The percentage of endothelial cells activated for endoglin expression in both aortic root and aortic arch in C57BL/6J mice. The endothelial expression of endoglin is significantly increased in mice fed with atherogenic diet compared with mice on chow diet. Atorvastatin treatment significantly decreased endoglin expression only in mice fed with atherogenic diet. Values are means ± SE, $n = 8$. *, statistical significance at $p < 0.05$.

The 8-week atorvastatin treatment at a dose of 10 mg/kg resulted in a significant decrease of total cholesterol and VLDL in mice fed the atherogenic diet. This hypolipidemic effect of statin treatment was accompanied by the significant reduction of endothelial expression of endoglin. On the contrary, atorvastatin treatment did not significantly affect cholesterol levels in mice on chow diet, which is consistent with Choudhury et al., who showed that simvastatin has no effect on cholesterol levels in C57BL/6J mice on chow diet (Choudhury et al. 2004). Simultaneously, endoglin expression was not significantly changed in these mice. These data suggest that endothelial expression of endoglin is upregulated by a high-cholesterol diet and decreased together with the hypolipidemic effect of atorvastatin in C57BL/6J mice. Taken together with our previous study, it appears that atorvastatin can affect endoglin expression by both hypolipidemic and pleiotropic effects.

At this time, however, we are not able to elucidate whether endoglin expression in vessel walls has positive or negative effects on atherogenesis. It has been demonstrated that endoglin participates in the TGF-β signaling cascade. The role of TGF-β in atherogenesis is controversial. Some authors suggest it is antiatherogenic (Grainger et al. 1994, 1995), whereas others provide evidence that it is atherogenic because elevated levels of TGF-β were found in vessel wall lesions (Majesky et al. 1991; Nikol et al. 1992). A similar situation seems to exist for endoglin. As described above, endoglin is expressed in human atherosclerotic aortas (Conley et al. 2000; Ma et al. 2000), and in this study its expression is increased by hypercholesterolemia. On the contrary, it has recently been demonstrated that the levels of endothelial nitric oxide synthase (eNOS) are dependent on the amount of endoglin, both in vivo and in vitro (Jerkic et al. 2004; Toporsian et al. 2005) and that endoglin upregulates eNOS expression at the transcriptional level, both in the absence and in the presence of exogenous TGF-β in endothelial cells (Santibanez et al. 2007). Endothelial nitric oxide synthase-derived NO is an endogenous vasodilatory molecule that regulates the tone of blood vessels and maintains an antithrombotic, antiproliferative, and antiapoptotic environment in the vessel wall (Sessa 2004). Alteration of eNOS function and NO availability in endothelium is strongly associated with the development of endothelial dysfunction, atherogenesis, and cardiovascular disease (Boger 2003).

In conclusion, we demonstrated that endothelial expression of endoglin is upregulated by hypercholesterolemia and decreased by the hypolipidemic effect of atorvastatin in C57BL/6J mice, suggesting that blood cholesterol levels

could partially affect endothelial expression of endoglin. Thus, we suggest that endoglin expression could be involved in atherogenesis and decreased by statin treatment. The precise importance of its participation in the atherogenic process, however, and its cooperation with TGF-β in advanced atherosclerotic lesions must still be elucidated.

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VI.

ATORVASTATIN HAS HYPOLIPIDEMIC AND ANTI-INFLAMMATORY EFFECTS IN APOE/LDL RECEPTOR-DOUBLE-KNOCKOUT MICE

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Atorvastatin has hypolipidemic and anti-inflammatory effects in apoE/LDL receptor-double-knockout mice

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Abstract

Statins are first-line pharmacotherapeutic agents for hypercholesterolemia treatment in humans. However the effects of statins in animal models of atherosclerosis are not very consistent. Thus we wanted to evaluate whether atorvastatin possesses hypolipidemic and anti-inflammatory effects in mice lacking apolipoprotein E/low-density lipoprotein receptor (apoE/LDLR-deficient mice). Two-month-old female apoE/LDLR-deficient mice (n=24) were randomly subdivided into 3 groups. The control group of animals (n=8) was fed with the western type diet (atherogenic diet) and in other two groups atorvastatin was added to the atherogenic diet at the dosage of either 10 mg/kg or 100 mg/kg per day for a period of 2 months. Biochemical analysis of lipids, ELISA analysis of monocyte chemotactic protein-1 (MCP-1) in blood, quantification of lesion size and expression of vascular cell adhesion molecule-1 (VCAM-1) and intercellular cell adhesion molecule-1 (ICAM-1) in the atherosclerotic lesion by means of immunohistochemistry and Western blot analysis were performed. The biochemical analysis showed that administration of atorvastatin (100 mg/kg/day) significantly decreased level of total cholesterol, lipoproteins (VLDL and LDL), triacylglycerol, and moreover significantly increased level of HDL. ELISA analysis showed that atorvastatin significantly decreased levels of MCP-1 in blood and immunohistochemical and Western blot analysis showed significant reduction of VCAM-1 and ICAM-1 expression in the vessel wall after atorvastatin treatment (100 mg/kg/day). In conclusion, we demonstrated here for the first time strong hypolipidemic and anti-inflammatory effects of atorvastatin in apoE/LDLR-deficient mice. Thus, we propose that apoE/LDLR-deficient mice might be a good animal model for the study of statin effects on potential novel markers involved in atherogenesis and for the testing of potential combination treatment of new hypolipidemic substances with statins.

Keywords: ApoE/LDLR-deficient mice; Hypolipidemic; Anti-inflammatory; Atorvastatin

Introduction

Statins (3-hydroxy-3-methylglutaryl-coenzyme A reductase inhibitors) are first-line pharmacotherapeutic agents for hypercholesterolemia treatment in humans. Upon decrease of elevated levels of low-density-lipoprotein cholesterol (LDL-cholesterol) levels, statins significantly reduces the incidence of coronary heart disease events and mortality in hypercholesterolemic patients. Statins are preferred in patients with combined dyslipidemia because they are more likely to reduce LDL-cholesterol levels to target values and also substantially lower triglyceride levels. However, if target levels are not reached, combination therapy should be considered (McKenney, 2001). Yet statins reduce cardiovascular events by only about 20–40%. Nonstatin therapies (either as monotherapy or in addition to statins) to reduce LDL-cholesterol by mechanisms that do not involve inhibition of 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase are likely to be useful for patients in need of LDL reduction; particularly those who either cannot

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take statins or respond only partially or not at all to statins alone (Shah, 2003).

Numerous animal species have been used to study the pathogenesis and potential treatment of the lesions of atherosclerosis. In recent years, there has been an explosion in the number of in vivo studies that is largely attributable to the use of mouse models to study atherogenic mechanism. Mice are highly resistant to atherosclerosis. Only when mice were fed a very high cholesterol, high fat diet that also contains cholic acid did their cholesterol levels rise and after many months on this diet they developed several layers of foam cells (Jawien et al., 2004). Genetic research and the application of transgene techniques and gene targeting in mice resulted in generation of a wide range of mouse strains that are much more suitable atherosclerotic models, including apoE-deficient mice, and LDLR−/− mice (Hofker and Breuer, 1998).

The apoE-deficient mouse is a well-established genetic mouse model of atherogenic hypercholesterolemia, in which mice spontaneously develop hypercholesterolemia and atherosclerosis on chow diet. The mice also develop widespread fibrous plaque lesions at vascular sites typically affected in human atherosclerosis and therefore represent an important model for studies of genetic and environmental influences on the atherosclerotic process (Reddick et al., 1994). The effect of statin treatment on atherogenesis in these mice seems to be time-dependent. Short-term administration of statin did not alter plasma lipids in apoE-deficient mice (Nachtigal et al., 2006b; Sparrow et al., 2001). Surprisingly, long-term administration of simvastatin elevated serum total cholesterol and increased aortic plaque area (Wang et al., 2002).

LDL receptor-deficient mice have been created to induce high plasma levels of LDL and IDL lipoproteins. These mice develop no, or only very small lesions on chow diet; however, robust lesions develop on the western type diet (Jawien et al., 2004). Still, the development of these lesions is slow and long-term experiments are needed. Effect of statin in this model of atherosclerosis is inconsistent. The administration of statins in these mice lowered total cholesterol and LDL-cholesterol levels and reduced aortic plaque area (Bisgaier et al., 1997; Wang et al., 2002). In contrast, Chen et al. (2002) showed that simvastatin did not alter plasma cholesterol or triglyceride levels in LDLR receptor-deficient mice. Thus, conflicting results are available for these mouse models of atherosclerosis regarding the effects of statins on atherogenesis.

More recently, apoE/LDLR-deficient mice have been created, representing a new mouse model that develops severe hyperlipidemia and atherosclerosis (Ishibashi et al., 1994). From papers previously published it is known that apoE/LDLR-deficient mice develop spontaneous hypercholesterolemia and atherosclerosis even on chow diet and addition of western type diet just increases cholesterol levels and potentiates atherogenesis in the same way as in other genetic mouse models of atherosclerosis (Bonthu et al., 1997; Ishibashi et al., 1994). Moreover it has been reported that, even on chow diet, the progression of atherosclerosis is more marked in these mice than in mice deficient for apoE or LDL receptor alone (Witting et al., 1999).

To the best of our knowledge the effect of statins on atherogenesis in apoE/LDLR-deficient mice has not been studied. Thus, the aim of this study was to evaluate whether atorvastatin possesses hypolipidemic and anti-inflammatory effects in these mice. We analyzed lipid profile and inflammatory markers in blood and together with expression of inflammatory markers and lesion size in the blood vessel wall.

Materials and methods

Animals

The Ethical Committee of the Faculty of Pharmacy, Charles University, approved the protocols of the animal experiments. The protocol of experiments was pursued in accordance with the directive of the Ministry of Education of the Czech Republic (No. 311/1997).

Experimental design

Two-month-old female apoE/LDLR-deficient mice on a C57BL/6J background (n=24) (Taconic Europe, Lille Skensved, Denmark) were randomly subdivided into 3 groups. Female mice were used in the experiment, because many papers published previously used female apoE/LDLR-deficient mice when testing potential antiatherogenic substances (Jawien et al., 2005, 2007; Olszanecki et al., 2005). Moreover it has been reported that female mice are more susceptible to atherogenesis than male littermates (Paigen et al., 1987; Rubanyi et al., 1997). Since we wanted to induce more severe atherogenesis we used female mice.

All mice were fed with the 3 different experimental diets for another 2 months with water ad libitum throughout the study. The control group of animals (n=8) was fed with the western type diet (atherogenic diet) containing 21% fat (11% saturated fat) and 0.15% cholesterol by weight. The same atherogenic diet and treatment period was used in other two groups where atorvastatin was added to the atherogenic diet at the dosage of either 10 mg/kg or 100 mg/kg per day. The design of the experiment was set according to the previous studies with apoE/LDLR-deficient mice where other antiatherogenic substances were tested (Chiwata et al., 2001; Jawien et al., 2005, 2006, 2007; Olszanecki et al., 2005). Moreover the dosage of atorvastatin used in this study was chosen according to our own and others' experiments with apoE-deficient or LDLR-deficient mice and statins where the doses ranged from 10 mg/kg/day up to 300 mg/kg/day (Zadelaar et al., 2007). Each mouse, in both statin groups, lived in a separate cage and obtained 4 g of food (in specially prepared pellets) daily. The food consumption was monitored every day. No differences in the food consumption were visible, either between animals of one experimental group or between experimental groups.

At the end of the treatment period, all animals were fasted overnight and euthanized. Blood samples were collected via cardiac puncture at the time of death. The aortas, attached to the top half of the heart, were removed and then immersed in OCT (Optimal Cutting Temperature) embedding medium (Leica, Prague, Czech Republic), snap frozen in liquid nitrogen and

stored at −80 °C, and descending aortas for Western blot analysis were frozen in liquid nitrogen and stored at −80 °C.

Biochemistry

Serum lipoprotein fractions were prepared using sodium chloride density gradient ultracentrifugation (Beckman TL 100, Palo Alto, CA, USA). The lipoprotein fractions were distinguished in the following density ranges: VLDL <1.006 g/ml; LDL <1.063 g/ml; HDL >1.063 g/ml. Total concentration and lipoprotein fraction concentration of cholesterol were measured enzymatically by conventional enzymatic diagnostic kits (Lachema, Brno, Czech Republic) and spectrophotometric analysis (cholesterol at 510 nm, triglycerides at 540 nm, ULTROSPECT III, Pharmacia LKB Biotechnology, Uppsala, Sweden).

ELISA analysis

Mouse soluble monocyte chemotactic protein-1 (MCP-1) was measured by quantitative sandwich enzyme immunoassay technique with using commercial diagnostic kit Mouse CCL2/JE/MCP-1 Quantikine ELISA Kit (R&D Systems, Inc., Minneapolis, MN, USA).

Oil Red staining

Frozen sections were used for the detection of lipids in the atherosclerotic lesions. Slides were air-dried at room temperature for 30 minutes and then stained for 30 minutes in a working solution of Oil Red (0.5 g of Oil Red was dissolved in 100 ml of isopropyl alcohol, and 60 ml of this solution was diluted with 40 ml of water and filtered). After being rinsed in running tap water the slides were stained in Gill's hematoxylin solution for 5 seconds and then washed thoroughly in running tap water for 1 minute. Slides were mounted with aqueous mounting medium.

Immunohistochemistry

Sequential tissue sectioning started in the mouse heart and continued until the aortic root containing semilunar valves together with the aorta appeared. The aortic sinus area was used for the histological and immunohistochemical evaluation because this is the reference tissue used for atherosclerosis evaluation in almost every study in mice. From this point on, serial cross-sections (7 μm) were cut on a cryostat and placed on gelatin-coated slides. Sections were air-dried and then slides were fixed for 20 minutes in acetone at −20 °C. After being rinsed in PBS (pH 7.4) the slides were incubated with anti avidin and anti biotin solutions (Vector Laboratories, USA). After blocking of non-specific binding sites with 10% normal goat serum (Sigma-Aldrich Chemie, Germany) in PBS solution (pH 7.4) for 30 minutes, slides were incubated with primary antibodies for 1 hour at room temperature. After a PBS rinse, the slides were developed with biotin-conjugated goat anti-rat Ig (diluted 1/400 in BSA) (BD Pharmingen™, California, USA) or biotin-conjugated goat anti-hamster Ig (diluted 1/600 in BSA) (BD Pharmingen™, California, USA) in the presence of 200 μg/ml normal mouse IgG (Dako, Denmark). Antibody reactivity was detected using HRP (horseradish peroxidase)-conjugated biotin-avidin complexes (Vector Laboratories, USA) and developed with diaminobenzidine tetrahydrochloride substrate (Dako, Denmark). Specificity of the immunostaining was assessed by staining with nonimmune isotype-matched immunoglobulins.

Primary antibodies included the following: monoclonal antibody Hamster Anti-Mouse CD54 (intercellular cell adhesion molecule ICAM-1, clone 3E2) diluted 1:200, monoclonal antibody Rat Anti-Mouse CD106 (vascular cell adhesion molecule VCAM-1, clone 429 (MVCAM.A)) diluted 1:100. Both antibodies were purchased from BD Pharmingen (California, USA).

Quantitative analysis of the immunohistochemistry

Stereological methods for the estimation of immunohistochemical staining of VCAM-1 and ICAM-1 were used as previously described (Nachtigal et al., 2002, 2004). In brief, the systematic uniform random sampling and the principle of the point-counting method were used for the estimation (Weibel, 1979). A total of 40 consecutive serial cross-sections were cut into 7 μm thick slices, which gave us 0.280 mm lengths of the vessel called the reference volume. This reference volume comprises several sections of the vessel containing semilunar valves in aortic root, and several sections of aortic arch (ascending part of the aorta). A systematic uniform random sampling was used in the reference volume. The first section for each immunohistochemical staining was randomly positioned in the reference volume and then each eighth section was used, thus five sections for each staining were used for the stereological estimation. The point-counting method was used and more than 100 test points per vessel, hitting immunostaining, were counted for an appropriate estimation (Gundersen et al., 1988). The estimated area is then:

$$\mathrm{est}A = a \times P$$

where the parameter a characterizes the test grid and P is the number of test points hitting either the atherosclerotic lesion or positive immunostaining.

Photo documentation and image digitizing from the microscope were performed with the Olympus AX 70, with a digital firewire camera Pixelink PL-A642 (Vitana Corp. Ottawa, Canada) with image analysis software NIS (Laboratory Imaging, Czech Republic). Stereological analysis was performed with a PointGrid module of the ELLIPSE software (ViDiTo, Kosice, Slovakia).

Western blot analysis

For Western blot analysis aortas from all groups of mice were homogenized in lysis buffer containing 10 mM Tris pH 7.4, 250 mM saccharose, 1 mM EDTA and protease inhibitor cocktail (Complete Mini, Roche Diagnostics GmbH, Mannheim, Germany). The homogenates were centrifuged at 2500 rpm for

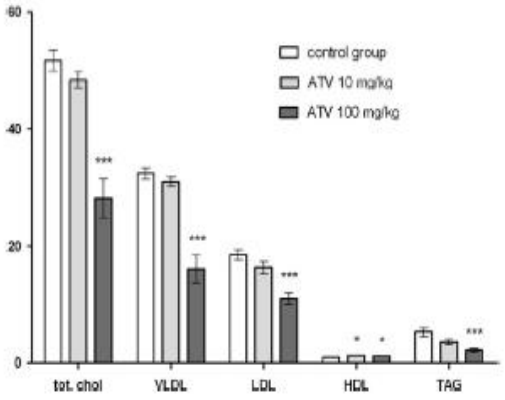

Fig. 1. Determination of lipid profile in apoE/LDLR-deficient mice. Atorvastatin treatment at dose 100 mg/kg/day significantly decreased total cholesterol, LDL-cholesterol, VLDL-cholesterol and TAG levels when compared with control mice. Moreover HDL-cholesterol levels were increased in both atorvastatin-treated groups. The treatment with the dose 10 mg/kg/day resulted only in mild and non-significant decrease of total cholesterol, VLDL, LDL and TAG. Values are means ± SEM, $n=8$. ***$P<0.001$, *$P<0.05$.

10 minutes and 10,000 rpm for 30 minutes at 4 °C. After determination of protein concentration in the supernatant by the BCA™ Protein Assay Kit (Pierce, Rockford, IL), samples (10 μg protein) were electrophoresed on 12% SDS-polyacrylamide gels. Proteins were then electrotransferred to a nitrocellulose membrane HYBOND™ -ECL™ (Amersham Biosciences, Uppsala, Sweden). After blocking of non-specific binding sites in 5% non-fat dry milk (= blocking buffer), membranes were incubated with Anti-Mouse VCAM-1 antibody (R&D Systems, Inc., Minneapolis, MN, USA; dilution 1:500 in 1% blocking buffer) overnight at 4 °C. Incubation with corresponding secondary rabbit anti-goat horseradish peroxidase-conjugated antibody (Pierce, Rockford, IL, USA; dilution 1:5000 in 1% blocking buffer) 60 minutes at room temperature was used for recognition of the primary antibody, followed by enhanced chemiluminiscence detection with a SuperSignal West Femto Maximum sensitivity Substrate (Pierce, Rockford, IL, USA). The membranes were subsequently exposed to FOMA® Blue Medical X-ray films (FOMA Bohemia, Hradec Kralove, Czech Republic). To quantify the bands of interest, exposed films were scanned with a GS-800 Calibrated Densitometer (Bio-Rad Laboratories, Hercules, CA, USA) and quantified using the QuantityOne imaging software (Bio-Rad Laboratories).

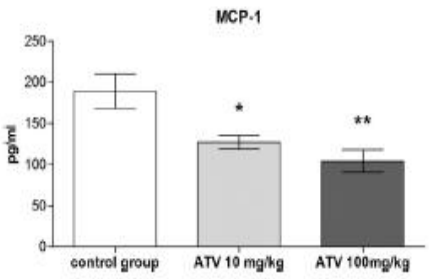

Fig. 2. ELISA analysis of MCP-1 in blood. Atorvastatin treatment significantly decreased levels of MCP-1 in both atorvastatin-treated mice when compared with control animals. Values are means ± SEM, $n=8$. *$P<0.05$, **$P<0.01$.

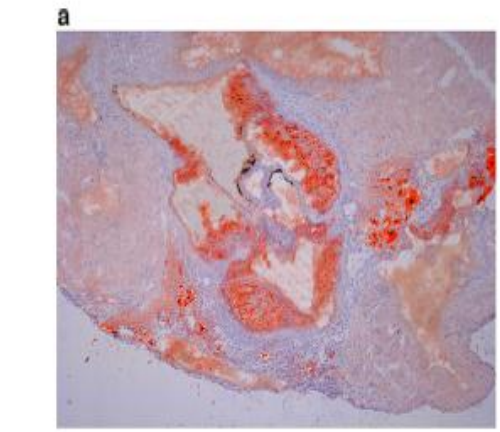

Fig. 3. Representative aortic sections of Oil Red staining from control group (a) and group treated with 100 mg/kg/day of atorvastatin (b). Atorvastatin treatment did not affect Oil Red area staining. Original magnification 40×.

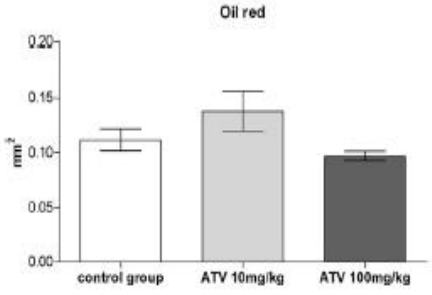

Fig. 4. Quantitative stereological analysis of Oil Red staining. Atorvastatin treatment did not significantly affect the area of Oil Red staining. Only mild and non-significant reduction was observed after treatment with atorvastatin at a dose of 100 mg/kg/day.

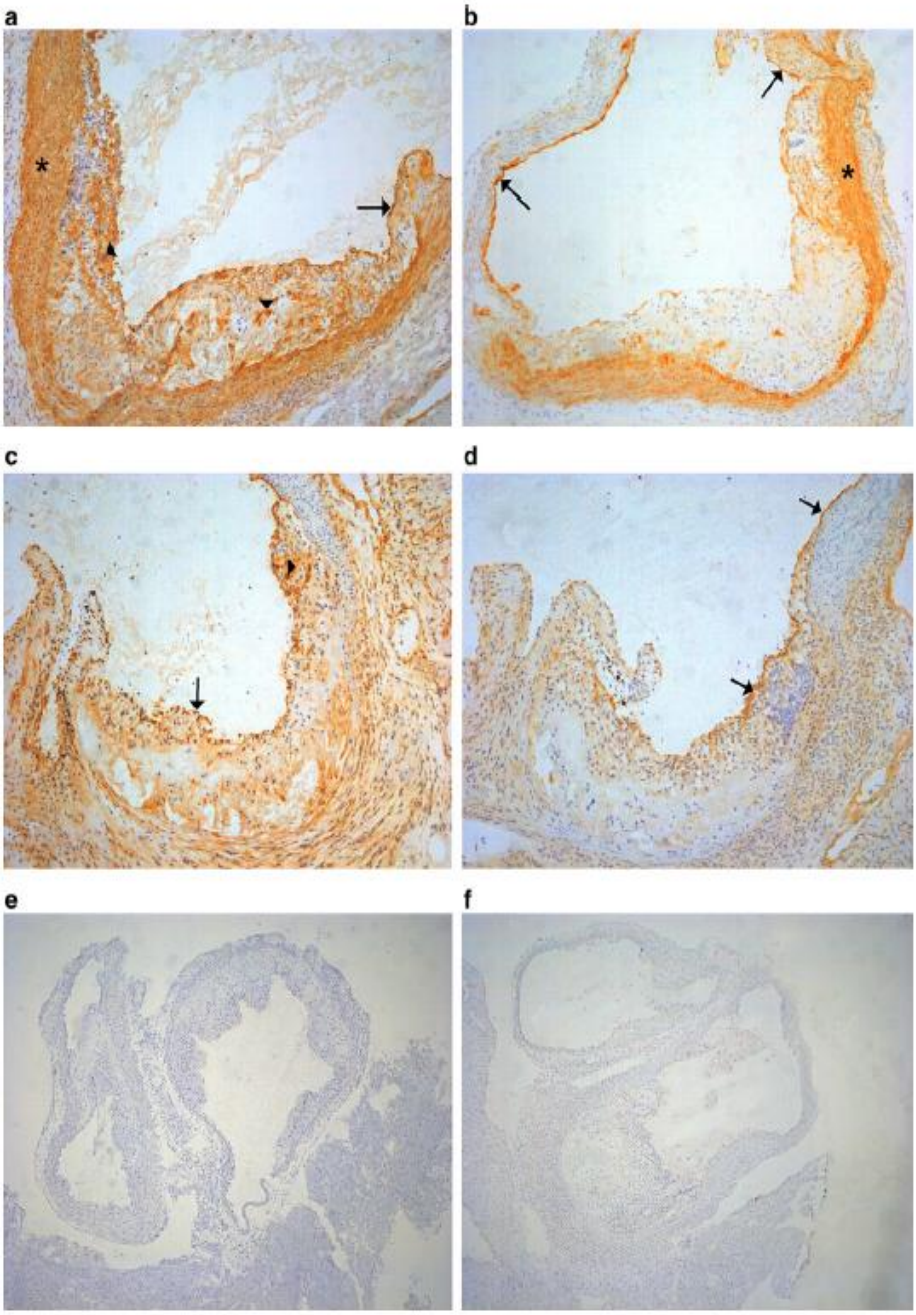

Fig. 5. Representative aortic sections showing immunohistochemical staining of VCAM-1 (a, b) and ICAM-1 (c, d) expression in aortic sinus. In the control group, the expression of VCAM-1 is strong in atherosclerosis lesion (arrowheads), in endothelium (arrows), and in vessel media in areas under the lesions (asterisk). (a). VCAM-1 expression is decreased after administration of 100 mg/kg/day of atorvastatin especially inside the lesion (b) in comparison with control mice (a). ICAM-1 expression in control group is marked in endothelium (arrows) and inside atherosclerotic lesion (arrowheads) (c). Also strong diminution of ICAM-1 staining after atorvastatin treatment at 100 mg/kg/day is visible predominantly inside the lesion (d) when compared with control mice (c). Endothelial expression of both VCAM-1 and ICAM-1 seemed to be unaffected by the atorvastatin treatment (b, d). Specificity of the immunostaining was assessed by omitting of primary antibody and staining with nonimmune isotype-matched immunoglobulins for VCAM-1 (e) and ICAM-1 (f). Original magnification 100×.

Statistical analysis

All values in the graphs are presented as a mean ± SEM of $n = 8$ animals. Statistical significance in the differences between groups was assessed by ANOVA test followed by Dunnett test for comparisons between treated and control group with the use of the GraphPad Prism software (version 4.0). P values of 0.05 or less were considered statistically significant.

Results

Biochemical analysis

Biochemical analysis of blood samples of apoE/LDLR-deficient mice showed that administration of atorvastatin at a dose of 100 mg/kg/day resulted in a significant decrease of total cholesterol (51.90±1.72 vs. 28.23±3.30 mmol/l, $P<0.001$) in comparison with control (non-treated) mice. Moreover the administration of this dose of atorvastatin resulted in a significant decrease of VLDL-cholesterol (32.41±0.97 vs. 16.10±2.41 mmol/l, $P<0.001$), LDL-cholesterol (18.59±0.81 vs. 11.00±1.04 mmol/l, $P<0.001$) and TAG levels (5.26±0.729 vs. 2.13±0.312 mmol/l, $P<0.001$) when compared with control mice (Fig. 1). In addition to these hypolipidemic effects atorvastatin treatment significantly increased levels of HDL-cholesterol at doses of 10 mg/kg/day (0.90±0.07 vs. 1.24±0.11 mmol/l, $P<0.05$), and 100 mg/kg (0.90±0.07 vs. 1.13±0.04 mmol/l, $P<0.05$) in comparison with control (non-treated) mice (Fig. 1). Conversely, treatment with the dose of 10 mg/kg/day resulted only in mild and non-significant decrease of total cholesterol, VLDL, LDL and TAG (Fig. 1).

ELISA analysis

ELISA analysis was aimed to reveal the effects of atorvastatin on pro-inflammatory monocyte chemotactic protein-1 (MCP-1) in blood. Atorvastatin treatment significantly decreased levels of MCP-1 in blood at the dose of 10 mg/kg/day (127.24±8.36 vs. 208.03±25.30 mmol/l, $P<0.05$), and 100 mg/kg/day (104.54±13.56 vs. 208.03±25.30 mmol/l, $P<0.01$) in comparison with control (non-treated) mice (Fig. 2).

Oil Red staining

The size of atherosclerotic lesions was quantified in all groups of animals by means of Oil Red staining (Fig. 3) and stereology (Fig. 4). Quantitative analysis of Oil Red stained aortic sections of atherosclerotic lesions indicated only very mild reduction in lesion size in the group treated with 100 mg/kg/day of atorvastatin compared with control mice (Fig. 4). Moreover, surprisingly, we observed mild increase in Oil Red staining area in mice treated with atorvastatin at 10 mg/kg/day (Fig. 4).

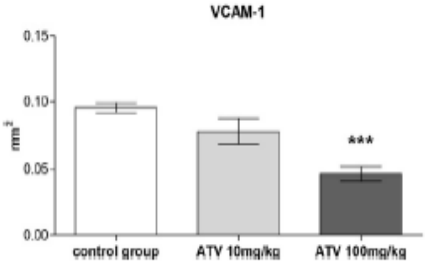

Fig. 6. The area of VCAM-1 expression in aortic root. Atorvastatin treatment (dose 100 mg/kg/day) resulted in a significant diminution of VCAM-1 expression compared with control mice. Values are means±SEM, $n=8$, ***$P<0.001$.

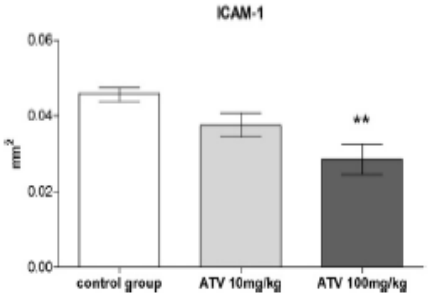

Fig. 7. The area of ICAM-1 expression in aortic root. The expression of ICAM-1 was significantly reduced in group treated with atorvastatin at a dose of 100 mg/kg/day in comparison with control mice. Values are means±SEM, $n=8$, **$P<0.01$.

Immunohistochemical staining of VCAM-1 and ICAM-1

In the control group, expression of VCAM-1 was observed in blood vessel intima (atherosclerotic lesion) and in endothelium covering atherosclerotic lesion as well as in endothelium outside the lesion (Fig. 5a). Moreover strong VCAM-1 expression was visible in smooth muscle cells of tunica media in areas under the lesions (Fig. 5a).

The staining patterns of VCAM-1 in atorvastatin-treated animals were similar. However strong reduction was notable especially inside the atherosclerotic lesion (Fig. 5b) and in some animals treated with 100 mg/kg/day even in vessel media under the lesion.

ICAM-1 expression in both control and atorvastatin-treated mice was visible in endothelium covering the lesion and outside it together with the expression inside the atherosclerotic lesion (Fig. 5c, d). No or very weak expression of ICAM-1 was visible in medial smooth muscle (Fig. 5c). Atorvastatin (100 mg/kg/day) treatment also decreased ICAM-1 expression predominantly inside the atherosclerotic plaque (Fig. 5d).

Stereological analysis of VCAM-1 and ICAM-1 expression

Stereological analysis of VCAM-1 staining confirmed significant diminution of VCAM-1 expression only in mice treated with the higher dose of atorvastatin (0.096±0.004 vs.

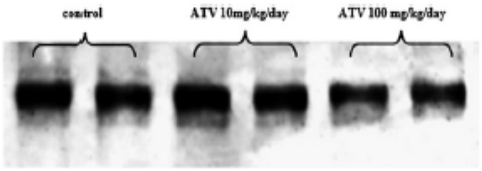

Fig. 8. Representative Western blot of VCAM-1 in the descending aortas from control mice and mice treated with atorvastatin (either 10 mg/kg/day or 100 mg/kg/day). Pooled samples from each group ($n=8$ in each group) were separated on a 12% polyacrylamide gel. Blots were detected with Anti-Mouse VCAM-1 antibody.

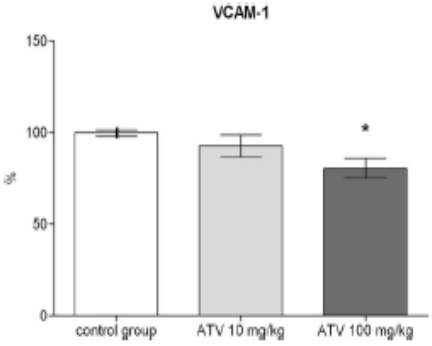

Fig. 9. Quantitative analysis of protein expression. VCAM-1 protein expression was significantly decreased in group treated with 100 mg/kg/day of atorvastatin compared with control mice. *$P<0.05$.

0.047 ± 0.006 mm^2, $P<0.001$) when compared with non-treated mice (Fig. 6).

Moreover significant decrease of ICAM-1 expression was confirmed in mice treated with 100 mg/kg/day (0.045 ± 0.002 vs. 0.028 ± 0.004 mm^2, $P<0.01$) in comparison to control mice (Fig. 7). Atorvastatin treatment with 10 mg/kg/day did not significantly decrease expression of either VCAM-1 or ICAM-1.

Western blot analysis

The protein expression of VCAM-1 in the descending aortas of all groups of mice was examined by Western blot analysis. Western blot analysis of pooled samples from each group is shown in Fig. 8. The group treated with atorvastatin at 100 mg/kg/day showed a lower band intensity of VCAM-1 compared with untreated mice. Densitometric analysis indicated that 100 mg/kg/day of atorvastatin significantly decreased VCAM-1 expression (80.39 ± 5.51 vs. $100.00\pm1.84\%$, $P<0.05$) compared with non-treated mice (Fig. 9).

Discussion

The advent of HMG-CoA reductase inhibitors, or statins, has revolutionized the treatment of hypercholesterolemia. Statins competitively inhibit HMG-CoA reductase, the enzyme that catalyzes the rate-limiting step in cholesterol biosynthesis. In addition a growing body of evidence suggests that statins exert beneficial vascular effects that are independent of their cholesterol-lowering potencies (Calabro and Yeh, 2005). In spite of the fact that statins are strong in decreasing of LDL-cholesterol and rosuvastatin also in increasing of HDL-cholesterol (Scharnagl and Marz, 2005; Scheen, 2006), combination treatment with other hypolipidemics with different mechanism of action is still required. The main goal of this strategy is to affect lipid parameters more completely and if possible to decrease the dose of statins to eliminate their possible adverse effects (Davidson and Robinson, 2007; Patel and Hughes, 2006). Thus statins are widely used in combination with ezetimibe (Pascual Izuel et al., 2005), fibrates (Jones, 2007) and niacin (Borges, 2005). Moreover new drugs or hypolipidemic substances are still under research. Therefore a good animal model where statin treatment would mimic the effects of statins in humans is required.

However statin effects in animal models of atherosclerosis are not very consistent. It has been demonstrated that statins exert some beneficial effects when administered to the most common models of atherogenesis rabbits and mice (Hernandez-Presa et al., 2002; Johnston et al., 2001). In rabbits, statins decrease cholesterol levels, lesion size and some inflammatory markers (Bustos et al., 1998; Nachtigal et al., 2005; Zhao et al., 2005). On the other hand rabbit atherogenesis (lesion development) is different from that in humans because rabbits usually develop fatty streaks or fibromuscular lesions but not advanced lesions, and moreover cholesterol overload in rabbits is necessary for the induction of atherosclerotic changes (Yanni, 2004). Therefore mouse models of atherosclerosis, mainly apoE-deficient mice and LDLR-deficient mice, are used (Bisgaier et al., 1997; Wang et al., 2002). However the effects of statins in these mouse models of atherosclerosis are controversial.

ApoE-deficient mouse is a well-established genetic mouse model of atherogenic hypercholesterolemia, which is similar to hyperlipoproteinemia type III in humans. These mice lack their principal ligand for the LDL receptor and, therefore, develop hypercholesterolemia and atherosclerosis on diet with normal fat content (Reddick et al., 1994). It has been demonstrated that statins do not decrease cholesterol levels in these mice but have positive anti-atherosclerotic effects, including decreased levels cholesterol in aorta (Sparrow et al., 2001), decreased expression of cell adhesion molecules (Monetti et al., 2007; Nachtigal et al., 2006b), and reduction of tissue factor expression (Monetti et al., 2007). Conversely, others have demonstrated that statins increase cholesterol levels in these mice, which is accompanied by no benefit in the vessel wall (Nachtigal et al., 2006a), or lead to increased atherosclerosis with no effect on endothelial function (Wang et al., 2002). Moreover it has been proposed that the enhanced hypercholesterolemia observed in apoE-deficient mice treated with simvastatin is caused by alterations in the assembly of VLDL by the liver, thus clearly demonstrating that apoE-deficient mice constitute a poor model to investigate potential effects of statins in the development of atherosclerosis (Fu and Borensztajn, 2006). Thus, we propose that the experimental design, especially the duration of treatment, the age of animals and the diet used, must be taken into consideration when studying statin effects in apoE-deficient mice.

LDL receptor-deficient mice represent another well-described and frequently used model of atherosclerosis. It has been demonstrated that statins decrease cholesterol levels in these mice and possess also anti-atherosclerotic effects and ameliorate endothelial dysfunction (Bisgaier et al., 1997; Wang et al., 2002). On the other hand it has been demonstrated that LDL receptor-deficient mice develop much smaller atherosclerotic lesions when compared with apoE-deficient mice, or that long-term cholesterol feeding up to 8 months must be used. Thus these mice are not a very suitable model for studying statin effects on well developed atherosclerotic lesions.

ApoE/LDLR-deficient mice that develop severe hyperlipidemia and atherosclerosis were introduced by Ishibashi et al. (1994). It has been reported that the progression of atherosclerosis is usually more marked in apoE/LDLR-deficient mice than in mice deficient in apoE alone (Witting et al., 1999). Thus, apoE/LDLR-deficient mice were considered as one of the best models to study the anti-atherosclerotic effect of several substances (Jawien et al., 2005, 2007; Olszanecki et al., 2005).

Since nothing is known about statin effects in apoE/LDLR-deficient mice, in the present study we used these mice to study the effects of two doses of atorvastatin (10 and 100 mg/kg/day) on lipid levels and inflammatory markers both in blood and vessel wall. We demonstrated significant decrease of total cholesterol, VLDL-, LDL-cholesterol and triglycerides but only after 100 mg/kg/day dose of atorvastatin when compared with non-treated mice. Prior studies in both mice and rats have suggested marked inactivation of HMG-CoA reductase inhibitors caused by robust P450 enzyme induction (Greenspan et al., 1988) and elevation of HMG-CoA reductase levels (Kita et al., 1980). This seems to be the explanation why atorvastatin treatment at 10 mg/kg/day did not result in hypolipidemic effect and why a higher dose of statins must be used in mice when compared with humans. Moreover we found a significant increase of HDL-cholesterol in both atorvastatin-treated groups. However, it has been demonstrated that statins decrease levels of HDL-cholesterol in LDL receptor-deficient mice (Bisgaier et al., 1997; Wang et al., 2002) or its levels are increased together with all other lipoproteins levels in apoE-deficient mice (Nachtigal et al., 2006a). Thus, atorvastatin effects on LDL and HDL levels demonstrated in this study are unique between mouse models of atherosclerosis and moreover similar to rosuvastatin effects on lipids described in humans (Scheen, 2006).

On the other hand it must be stated that there are differences between cholesterol metabolism in mice and humans. Mice possess a major amount of cholesterol in VLDL but in humans the major amount of cholesterol is in LDL fraction. Also double deficit of apoE lipoprotein and LDL receptor has not been described in humans; thus a possible hypolipidemic mechanism of action in these mice must be different from hypolipidemic effects of statins in humans. In spite of these differences the final effects of atorvastatin treatment in these mice somehow mimic the effects of latest used statins on lipids in humans (Scheen, 2006).

Another beneficial effect of the statins may be related to their capacity to inhibit expression of pro-inflammatory chemokines by cells. MCP-1, a member of the C–C chemokine family is a potent chemotactic factor for monocytes and has been shown to play a fundamental role in the initiation and progression of atherosclerotic lesions in hyperlipidemic mice (Aiello et al., 1999). In the present study atorvastatin treatment resulted in a significant decrease of this inflammatory marker in blood serum. Similar effects of statin treatment were described in mice (Han et al., 2005; Kleemann et al., 2003) and humans (Mulhaupt et al., 2003).

Atorvastatin treatment at 100 mg/kg/day, however, resulted only in a very mild decrease of Oil Red staining in the aorta, suggesting that lesion size was not affected. Moreover atorvastatin treatment at 10 mg/kg/day resulted in mild non-significant increase in lesion size when compared with control mice. This effect could reflect that the 10 mg/kg/day dose of atorvastatin was not sufficient to affect either cholesterol or inflammatory marker expression and thus the slight increase could indicate minor progression of atherogenesis in mice treated with this dose of atorvastatin. However, further studies are necessary for the clarification of these negative results of atorvastatin treatment on plaque size.

We also studied the expression of inflammatory markers in the vessel wall. Members of the immunoglobulin superfamily of endothelial adhesion molecules, vascular cell adhesion molecule (VCAM-1) and intercellular cell adhesion molecule (ICAM-1), strongly participate in leukocyte adhesion to the endothelium. The expression of VCAM-1 and ICAM-1 was studied by several authors in rabbit and mouse models of atherosclerosis (Iiyama et al., 1999; Li et al., 1993; Nakashima et al., 1998). It has been shown that VCAM-1 and ICAM-1 are detected in the regions predisposed to atherosclerotic lesion formation in normocholesterolemic rabbits, and the expression of both molecules is upregulated by a high-cholesterol diet in rabbits and mice (Iiyama et al., 1999). In this study the expression of VCAM-1 was strong in endothelium, inside the lesion and moreover in vessel media under the atherosclerotic lesions. ICAM-1 was detected predominantly in endothelium and in atherosclerotic lesion. These results are consistent with CAM expression described by Iiyama et al. (1999). Immunohistochemical staining showed that atorvastatin treatment (100 mg/kg/day) resulted in decreased expression of both ICAM-1 and VCAM-1 predominantly inside atherosclerotic lesion (e.g. in macrophages, T lymphocytes, and smooth muscle cells) and partially in vessel media under the lesion for VCAM-1 (smooth muscle cells) but not in endothelium. We cannot address which cells inside the plaque were responsible for the decreased expression of both adhesion molecules because we did not perform double immunostaining of VCAM-1 and ICAM-1 with markers of these cells. The reduced expression of VCAM-1 and ICAM-1 only in plaque cells but not in endothelium could reflect that endothelial expression of both VCAM-1 and ICAM-1 is not so strongly correlated with cholesterol levels in mice as was demonstrated previously (Nachtigal et al., 2006a; Zibara et al., 2000). Reduced expression of VCAM-1 and ICAM-1 in the vessel wall was confirmed by stereological analysis and also by Western blot analysis of VCAM-1.

Thus these results clearly demonstrate strong anti-inflammatory effects of atorvastatin treatment at a dose of 100 mg/kg/day in apoE/LDLR-deficient mice, as has also been demonstrated in humans (Tsiara et al., 2003).

The above mentioned results strongly propose that apoE/LDLR-deficient mice might be used for the study of potential novel antiatherogenic substances with statins where one can monitor possible benefit of combination treatment of statins and new substances being tested.

In conclusion, to the best of our knowledge, we demonstrated here for the first time strong hypolipidemic and anti-inflammatory

effects of atorvastatin in apoE/LDLR-deficient mice. Only treatment with the higher dose of atorvastatin (100 mg/kg/day) resulted in a significant decrease of lipid parameters including LDL-cholesterol and triglycerides with concurrent increase in HDL-cholesterol. Anti-inflammatory effects included decrease in serum levels of MCP-1 and strong reduction of VCAM-1 and ICAM-1 expression in the vessel wall. Thus, we propose that apoE/LDLR-deficient mice might be a good animal model for the study of statin effects on potential novel markers involved in atherogenesis. Moreover, in spite of probably different mechanism of action of atorvastatin in these mice when compared with humans, the final effects in this animal model mimic the effects of statins in humans. Thus, we propose that these mice can be used for the testing of potential combination treatment of new hypolipidemic substances with statins.

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VII.

ATORVASTATIN INCREASES ENDOGLIN, SMAD2, P-SMAD2/3 AND ENOS EXPRESSION IN APOE/LDLR-DEFICIENT MICE

Nachtigal P, **Pospisilova N,** Vecerova L, Micuda S, Brcakova E, Pospechova K, Semecky V: Atorvastatin increases endoglin, SMAD2, p-SMAD 2/3 and eNOS expression in apoE/LDLR-deficient mice 2008. *Journal of Atherosclerosis and Thrombosis,* acceptable for publication

Atorvastatin increases endoglin, SMAD2, P-SMAD2/3 and eNOS expression in apoE/LDLr-deficient mice

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Abstract

Endoglin is a homodimeric transmembrane glycoprotein that has been demonstrated to affect transforming growth factor β (TGF-β) signaling and endothelial nitric oxide synthase (eNOS) expression by affecting SMAD proteins *in vitro*. Thus, in this study we stepped forward to elucidate whether endoglin is co-expressed with SMAD2, phosphorylated SMAD2/3 proteins and eNOS *in vivo* in atherosclerotic lesions in ApoE/LDLR double knockout mice. In addition, we sought whether endoglin expression as well as the expression of SMAD2, phosphorylated SMAD2/3 and eNOS is affected by atorvastatin treatment.

Two-month–old female ApoE/LDLR double knockout mice were divided into two groups. The control group was fed with the western type diet whereas in the atorvastatin group, atorvastatin at dose 100 mg/kg per day was added to the same diet. Immunohistochemical and western blot analysis of endoglin, SMAD2, phosphorylated SMAD2/3 and eNOS expressions in aorta were performed.

The biochemical analysis showed that administration of atorvastatin significantly decreased level of total cholesterol, VLDL, LDL, TAG, and significantly increased level of HDL cholesterol. Fluorescence immunohistochemistry showed endoglin co-expression with SMAD2, phosphorylated SMAD2/3 and eNOS in aortic endothelium covering atherosclerotic lesions in both control and atorvastatin treated mice. Western blot analysis demonstrated that atorvastatin significantly increased expression of endoglin, SMAD2, phosphorylated SMAD2/3, and eNOS in mice aorta.

In conclusion, these findings suggest, that endoglin might be interesting marker of endothelial dysfunction and/or atherogenesis which is upregulated by statins implicating potential beneficial role of endoglin and its pathway in atherosclerosis.

Keywords: endoglin; SMAD2; P-SMAD2/3; eNOS; atorvastatin; apoE/LDLr-deficient mice

Introduction

Endoglin is a 190-kDa homodimeric transmembrane glycoprotein composed of 95-kDa disulfide-linked subunits (Guerrero-Esteo 1999). The primary sequence of human endoglin is composed of an extracellular domain of 561 amino acids, a single transmembrane region, and a cytoplasmic tail (Zhang 1996). Mutations in the gene encoding endoglin have been linked to the human disease: hereditary hemorrhagic telangiectasia type 1 (HHT1), an autosomal dominant inherited vascular disorder (Behr-Roussel 2000). Endoglin is a part of the transforming growth factor-β (TGF-β) receptor cascade and it is known as a type III TGF-β receptor. Endoglin forms complexes with heteromeric complexes of type I and type II serine/threonine kinase receptors (TβRI and TβRII), respectively and has been postulated to affect TGF-β1 signaling (Conley 2000). Activation of TGF-β signaling results in activation and translocation of the SMAD family of proteins to the nucleus to participate in regulating gene expression (Lebrin 2005). We previously demonstrated that endoglin is expressed by aortic vessel endothelium in normo- and hypercholesterolemic mice (Nachtigal 2006, Nachtigal 2007). Moreover, endoglin expression was also detected in human and porcine atherosclerotic lesions (Behr-Roussel 2000, Conley 2000, Piao 2006).

The endothelium plays a dual role in the regulation of the vasomotor tone. It produces and releases both relaxing and constricting factors. The main vasorelaxing factor produced by endothelial cells (EC) is nitric oxide (NO) (Lahera 2007). NO possesses several important biological effects including the inhibition of cell adhesion molecules expression and thus leukocyte adhesion to endothelium, inhibition of platelet aggregation and activation and inhibition of smooth muscle proliferation (Sessa 2004). NO synthesis by endothelium is maintained by endothelial nitric oxide synthase (eNOS) which is constitutively expressed but also affected by different stimuli including hypoxia, shear stress and LDL. It has been demonstrated that alteration of eNOS expression is related to the development and progression of atherosclerosis (Mungrue 2003).

Lipid-lowering drugs offer one of the most effective therapeutic approaches used in clinical practice for the prevention and treatment of atherosclerosis. Statins, a well known class of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors, are active in the primary and secondary prevention of coronary heart disease and are the drugs most widely used for these purposes (Schonbeck 2004). Regarding endoglin regulatory pathway, it has been demonstrated that endoglin expression by endothelium in non-atherosclerotic vessels in mice is affected by atorvastatin treatment (Nachtigal 2006) and that statins can increase eNOS expression by endothelium (Schalkwijk 2007).

Recently it has been demonstrated that endoglin expression correlates with eNOS expression and NO-dependent vasodilatation (Jerkic 2004), and that endoglin increases eNOS expression by modulating SMAD2 protein levels in endothelial cells *in vitro* (Santibanez 2007). However, to the best to our knowledge there are no available data demonstrating that endoglin co-localizes with SMAD2 and eNOS in endothelial cells *in vivo*. Moreover, it is unsure whether the proteins of this pathway could be affected by statin treatment in atherosclerotic lesions.

Thus, in this study we wanted to elucidate whether endoglin is co-expressed with SMAD2, phosphorylated SMAD2/3 proteins and eNOS *in vivo* in advanced atherosclerotic lesions in ApoE/LDLR double knockout mice by means of fluorescence immohistochemistry and whether endoglin expression and expression of SMAD2, phosphorylated SMAD2/3 and eNOS as well is affected by atorvastatin treatment.

Material and methods

Animals

Two-month old female ApoE/LDLR double knockout mice on a C57BL/6J background (n=16) (Taconic Europe, Lille Skensved, Denmark) were randomly subdivided into two groups.

All mice were fed with the different experimental diets for further 2 months with water *ad libitum* throughout the study. The control group of animals (n=8) was fed the western type diet (atherogenic diet) containing 21% fat (11% saturated fat) and 0.15% cholesterol by weight. The same atherogenic diet and treatment period was used in atorvastatin treated mice (n=8) where atorvastatin was added to the diet at a dose of 100 mg/kg per day. The dosage of atorvastatin used in this study was chosen according to both our own and others' experiments with apoE – deficient or LDLr – deficient mice and statins, where the doses ranged from 10 mg/kg/day up to 300 mg/kg/day (Zadelaar 2007). Each mouse, in both groups, lived in a separate cage obtaining 4 g of food (in specially prepared pellets) daily. The food consumption was monitored every day. No differences in the food consumption were visible, either between animals of one experimental group or between experimental groups.

At the end of the treatment period, all animals were fasted overnight and euthanized. Blood samples were collected via cardiac puncture at the time of death. The aortas, attached to the top half of the heart, were removed and then immersed in OCT (Optimal Cutting Temperature) embedding medium (Leica, Prague, Czech Republic), snap frozen in liquid nitrogen and stored at -80°C. Descending aortas for western blot analysis were frozen in liquid nitrogen and stored at -80°C.

Biochemistry

Serum lipoprotein fractions were prepared using NaCl density gradient ultracentrifugation (Beckman TL 100, Palo Alto, CA). The lipoprotein fractions were distinguished in the following density ranges: very low density lipoprotein (VLDL) < 1.006 g/ml; low density lipoprotein (LDL) < 1.063 g/ml; and high density lipoprotein (HDL) > 1.063 g/ml. Total concentration and lipoprotein fraction concentration of cholesterol were assessed enzymatically by conventional diagnostic kits (Lachema, Brno, Czech Republic) and spectrophotometric analysis (cholesterol at 510 nm, triglycerides, at 540 nm wavelength), (ULTROSPECT III, Pharmacia LKB Biotechnology, Uppsala, Sweden).

Immunohistochemistry

Sequential tissue sectioning started in the mouse heart until the aortic root containing semilunar valves together with the aorta appeared. From this point on, serial cross-sections (7μm) were cut on a cryostat and placed on gelatin-coated slides. Sections were air-dried and then slides were fixed for 20 minutes in acetone at -20°C. For the detection of endoglin expression slides were rinsed in PBS (pH 7.4) and then incubated with anti avidin and anti biotin solutions (Vector Laboratories, USA). After blocking of nonspecific binding sites with 10% normal goat serum (Sigma-Aldrich Chemie, Germany) in PBS solution (pH 7.4) for 30 min, slides were incubated with primary antibodies for 1 hour at room temperature. After a PBS rinse, the slides were developed with biotin-conjugated goat anti-rat Ig (diluted 1/400 in BSA) (BD Pharmingen™, California, USA) in the presence of 200 μg/ml normal mouse IgG (Dako, Denmark). Antibody reactivity was detected using HRP (Horseradish

peroxidase)-conjugated biotin-avidin complexes (Vector Laboratories, USA) and developed with diaminobenzidine tetrahydrochloride substrate (Dako, Denmark).

For the double fluorescence staining goat anti-rat secondary antibody marked with green fluorochrome (CY2) was used (diluted 1/100 in BSA) to detect endoglin. Goat anti-rabbit secondary antibody marked with red fluorochrome (CY3) was used (diluted 1/100 in BSA) for the detection of SMAD2, SMAD2/3 and eNOS. The specificity of the immunostaining was assessed by staining with nonimmune isotype-matched immunoglobulins.

Primary antibodies included the following: monoclonal antibody Rat Anti-Mouse Endoglin CD 105 (diluted 1:50) purchased from BD Pharmingen (California, USA), Rabbit polyclonal antibodies to phosphorylated P-SMAD2/3 (diluted 1:100) and eNOS (diluted 1:100), obtained from SantaCruz Biotechnology, Inc., (California, USA) and Rabbit polyclonal antibody directed to SMAD2 (diluted 1:30), obtained from Abcam (Cambridge, UK).

Photo documentation and image digitizing from the microscope were performed with the Olympus AX 70 light and fluorescence microscope, with a digital firewire camera Pixelink PL-A642 (Vitana Corp. Ottawa, Canada) and with VDS Vosskuehler CD-1300QB monochromatic camera for the fluorescence with image analysis software NIS (Laboratory Imaging, Czech Republic).

Western Blot Analysis

Western blot analysis was performed as described previously (Nachtigal 2008). Briefly, descending aortas from both groups of mice were homogenized in lysis buffer containing 10 mM Tris, 250 mM saccharose, 1 mM EDTA and protease inhibitor cocktail (Complete Mini, Roche Diagnostics GmbH, Mannheim, Germany). The homogenates were centrifuged at 2,500 rpm for 10 minutes and 10.000 rpm for 30 minutes at 4 °C. The protein concentration in the supernatant was determined with the BCA Protein Assay Kit (Pierce, Rockford, IL). Samples (10 μg protein) were incubated with sample buffer at room temperature for 30 minutes and separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS–PAGE) on 10% resp. 7.5% polyacrylamide gels. After the proteins were transferred to a nitrocellulose membrane (Bio-Rad, Hercules, CA, USA), they were blocked for 1 h with 5% nonfat dry milk in Tris-buffered saline containing 0.05% Tween 20 (TBST). The membrane was then incubated with primary antibodies (same as for immunohistochemistry – see above) at the following concentrations: endoglin (90-95 kDa) and eNOS (140 kDa) at 1:500, SMAD2 (58 kDa) and P-SMAD2/3 (52 kDa) at 1:300, secondary rabbit anti-goat horseradish peroxidase-conjugated antibody at 1:5000 and horseradish peroxidase-linked donkey anti-rabbit immunoglobulin G (GE Healthcare, Prague, CZ) at 1:2500 or 1:1000. After washing with TBST buffer, the membranes were developed using SuperSignal West Femto Maximum sensitivity Substrate (Pierce, Rockford, IL). The membranes were subsequently exposed to Hyperfilms (GE Healthcare, Prague, CZ). To quantify the bands of interest, exposed films were scanned with a ScanMaker i900 (UMAX, Prague, CZ) and quantified using the QuantityOne imaging software (Bio-Rad Laboratories). Equal loading of proteins onto the gel was confirmed by immunodetection of beta-actin (Anti-beta-actin antibody, Sigma, USA – diluted at 1:5000).

Statistical analysis

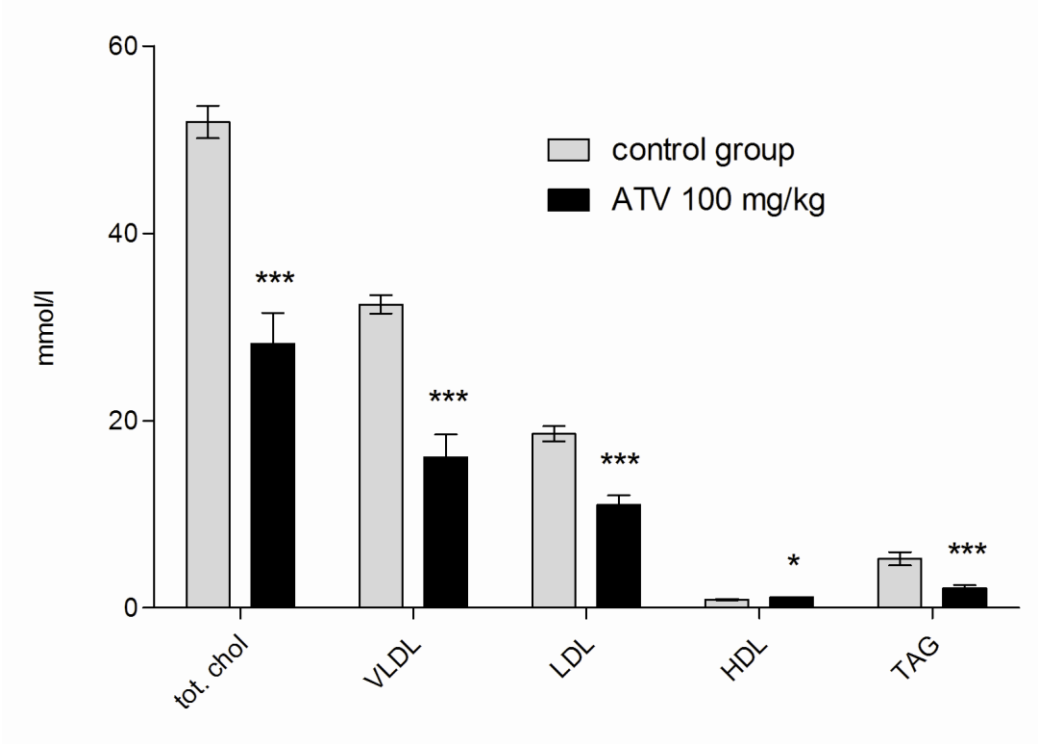
All values in the graphs are presented as a mean ± SEM of n=8 animals. Statistical significance in the differences between groups was assessed by t-test with the use of the GraphPad Prism software (version 5.0). P values of 0.05 or less were considered statistically significant.

Results

Biochemistry

Biochemical analysis of blood samples of ApoE/LDLR double knockout mice showed that the administration of 100 mg/kg/day of atorvastatin resulted in a significant decrease of total cholesterol (51.9 ± 1.7 vs. 28.2 ± 3.3 mmol/l, $P < 0.001$) in comparison with control (non-treated) mice. Moreover the administration of atorvastatin also resulted in a significant decrease of VLDL cholesterol (32.4 ± 1.0 vs. 16.1 ± 2.4 mmol/l, $P < 0.001$), LDL cholesterol (18.6 ± 0.8 vs. 11.0 ± 1.0 mmol/l, $P < 0.001$) and TAG levels (5.3 ± 0.7 vs. 2.1 ± 0.3 mmol/l, $P < 0.001$) when compared with control mice (Fig.1). In addition to these hypolipidemic effects, atorvastatin treatment significantly increased levels of HDL cholesterol (0.9 ± 0.07 vs. 1.1 ± 0.04 mmol/l, $P < 0.05$) in comparison with control (non-treated) mice (Fig. 1).

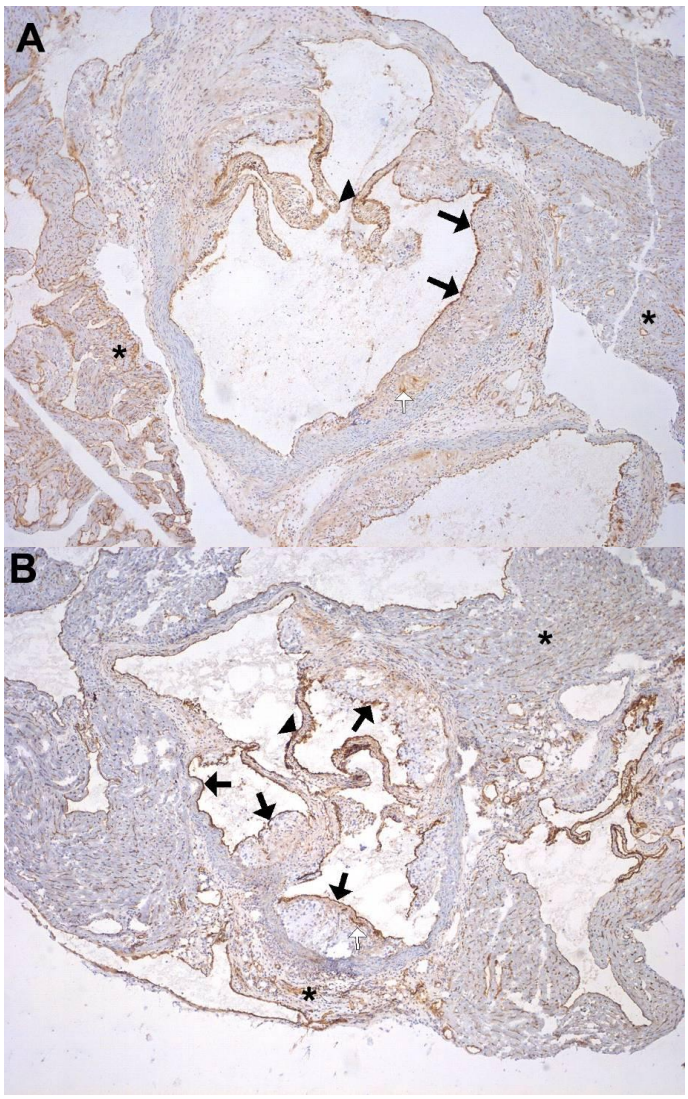
Fig. 1. Determination of lipid profile in ApoE/LDLR double knockout mice. Atorvastatin treatment significantly decreased total cholesterol, LDL cholesterol, VLDL cholesterol and TAG levels when compared with control mice. Moreover HDL cholesterol levels were increased in atorvastatin treated groups. Values are means ± SEM, n = 8. ***p< 0.001, *p< 0.05.



Immunohistochemical staining of endoglin in apoE/LDLR double knockout mice.

The expression of endoglin in aortic sinus of ApoE/LDLR double knockout mice was visible predominantly in endothelium covering the atherosclerotic lesion, endothelium of aortic valves, outside the lesion and in the capillaries of surrounding myocardium (**Fig. 2A, B**). However, in some vessels, weak staining was also visible in atherosclerotic lesion suggesting the additional expression of endoglin by other intimal cells. The staining pattern of endoglin expression was similar in both control and atorvastatin treated mice, however stronger staining intensity of endoglin predominantly in endothelium covering atherosclerotic lesion was seen in atorvastatin treated mice (**Fig. 2A, B**).

Fig. 2. Endoglin expression in aorta of control (A) and atorvastatin (B) treated apoE/LDLr-deficient mice. The expression of endoglin was visible in endothelium covering the atherosclerotic lesion (arrows), endothelium of aortic valves (arrowhead) outside the lesion, and in capillaries in surrounding myocardium (asterisk). Weak expression was also detected in intimal cells of atherosclerotic plaque (white arrow). The staining of endoglin especially in endothelium covering atherosclerotic lesion was stronger in atorvastatin treated animals (B) when compared with controls (A). The slides were counterstained with hematoxyline. Original magnification 40x.



Colocalization study of endoglin with SMAD2, phosphorylated SMAD2/3 and eNOS in ApoE/LDLR double knockout mice.

Double fluorescence staining of endoglin with SMAD2, phosphorylated SMAD2/3 and eNOS was performed. The expression of SMAD2 was detected by anti SMAD2 antibody, which should detect the inactivated (non-phosphorylated) form of SMAD2 in cells. The expression of phosphorylated SMAD2/3 was detected by anti SMAD2/3 antibody, which should detect activated (phosphorylated) form of SMAD2/3. The results revealed strong co-expression of endoglin with SMAD2 (**Fig. 3**), phosphorylated SMAD2/3 (**Fig. 4**), and eNOS (**Fig. 5**) in aortic vessel endothelium covering the atherosclerotic plaque in both control and atorvastatin treated mice. SMAD2 and phosphorylated SMAD2/3 expression was also detected in intimal cells of atherosclerotic plaque (**Fig. 4**); however no colocalization with endoglin was detected in this area. No significant differences in colocalization staining patterns of all proteins were visible in control and atorvastatin treated mice (data not shown).

Fig. 3. Co-expression of endoglin and SMAD2 in aorta of control apoE/LDLr-deficient mice. Endoglin (green) and SMAD2 (red) co-expression was detected only in aortic vessel endothelium covering the atherosclerotic plaque (arrows). No co-localization was detected inside the atherosclerotic lesion. Hoechst dye (blue) was used for the counterstaining. Original magnification 100x.

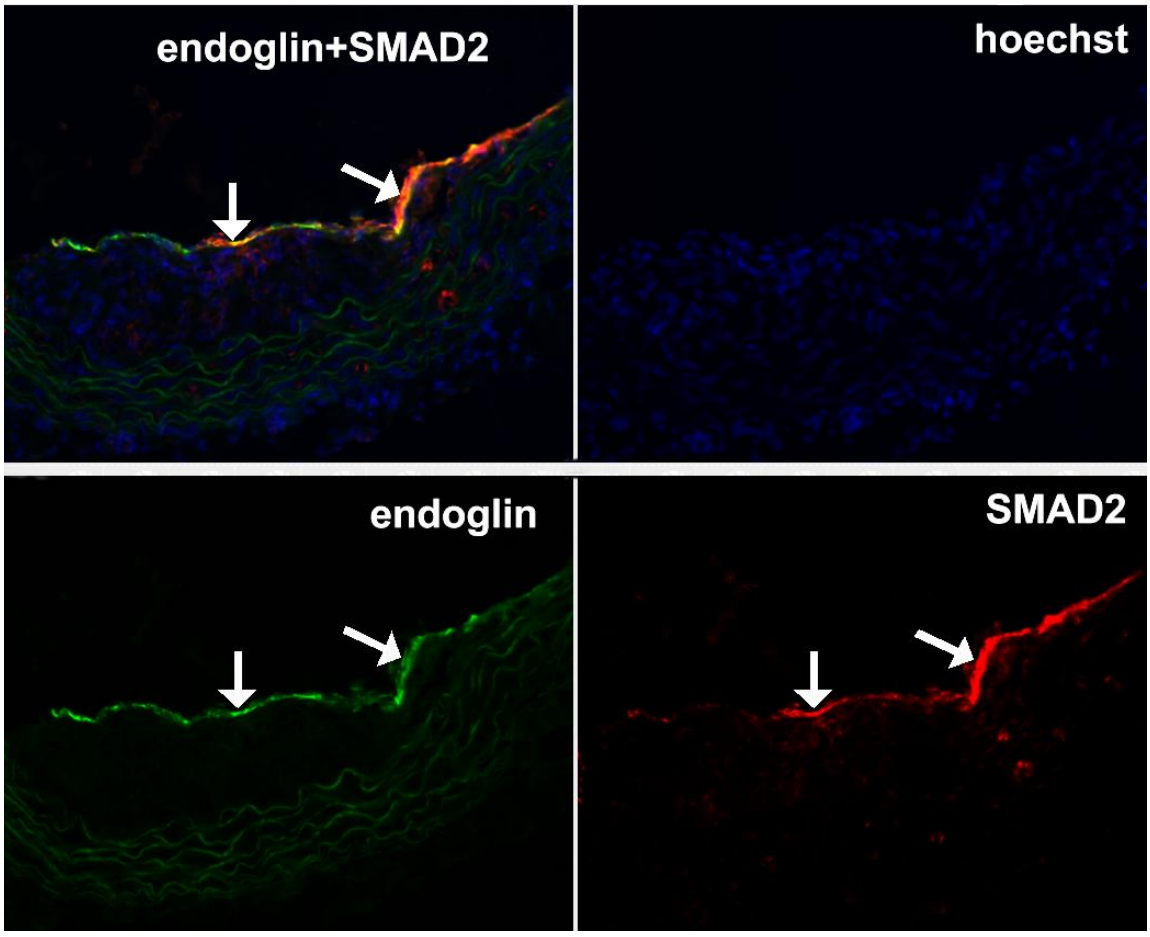


Fig. 4. Co-expression of endoglin and P-SMAD2/3 in aorta of control apoE/LDLr-deficient mice. Endoglin (green) and SMAD2/3 (red) co-expression was detected only in aortic vessel endothelium covering the atherosclerotic plaque (arrows). Strong P-SMAD2/3 staining is also visible in atherosclerotic intima and partially vessel media (arrowhead), however no co-localization was detected in this area. Hoechst dye (blue) was used for the counterstaining. Original magnification 100x.

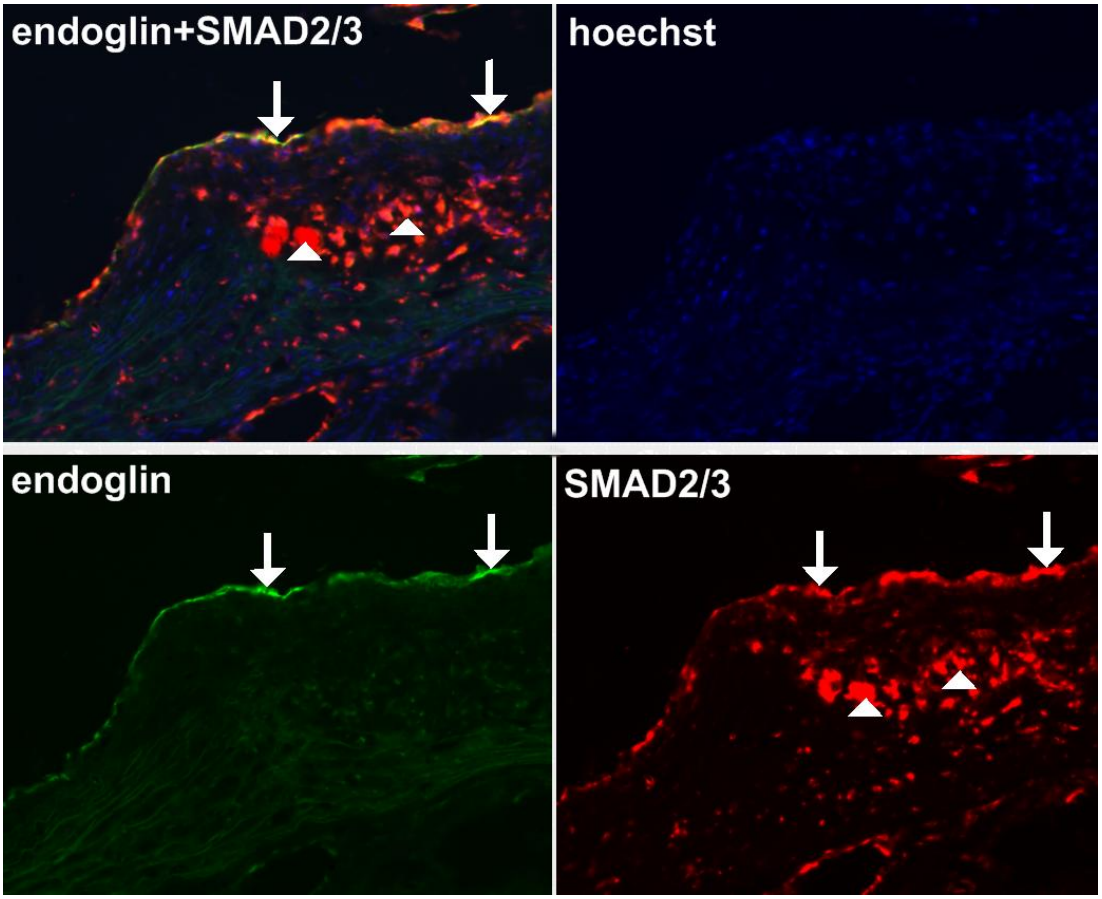
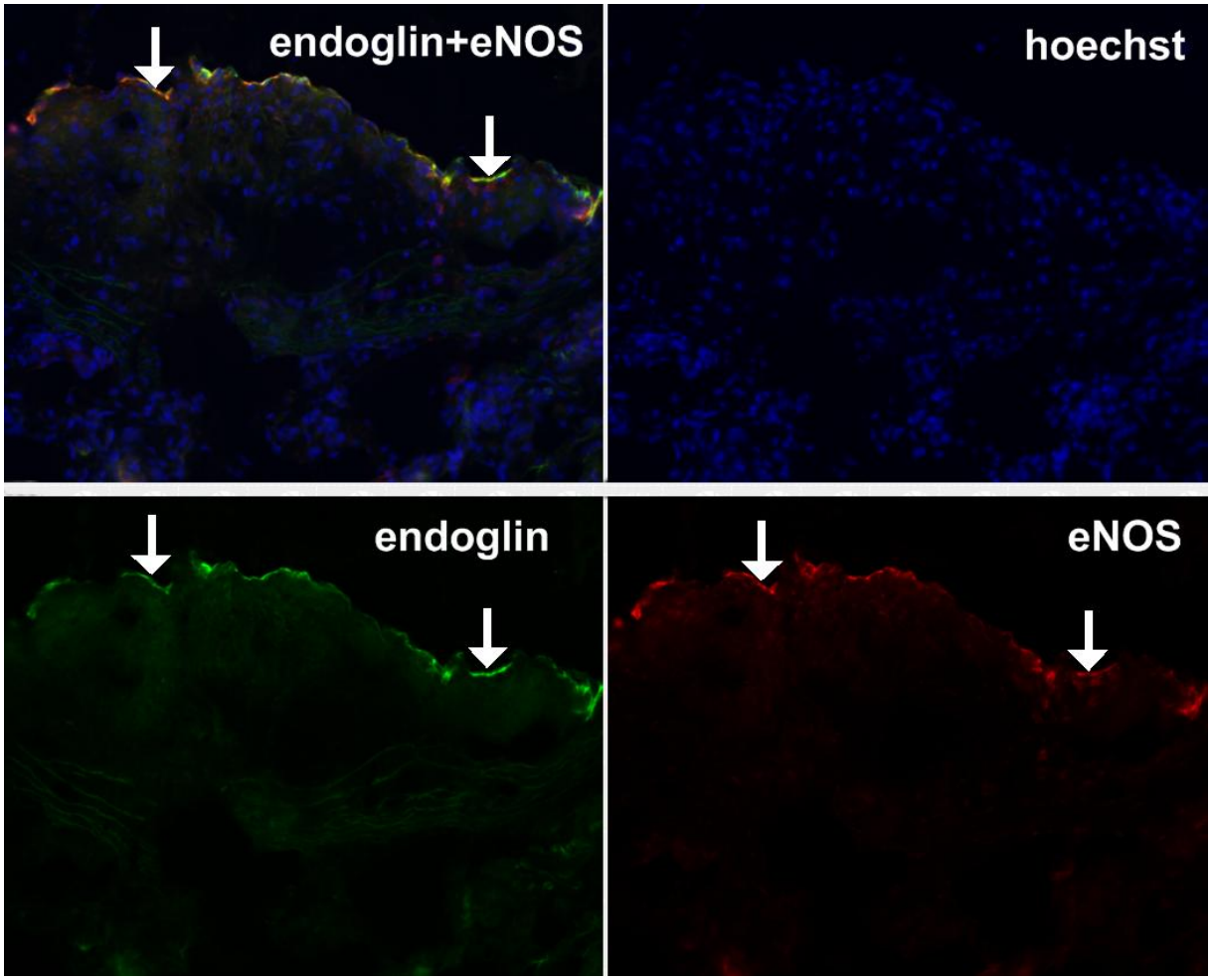


Fig. 5. Co-expression of endoglin and eNOS in aorta of control apoE/LDLr-deficient mice. Endoglin (green) and eNOS (red) co-expression was detected only in aortic vessel endothelium covering the atherosclerotic plaque (arrows). Hoechst dye (blue) was used for the counterstaining. Original magnification 100x.



Western Blot Analysis

The protein expression of eNOS, SMAD2, P-SMAD2/3, and endoglin in the mice descending aortas was examined by Western blot. As shown in **figures 6-9**, atorvastatin treatment (100 mg/kg orally for 2 months) induced expression of all measured proteins to 132-171% ($P < 0.001$) of values detected in control untreated animals. Equal loading of proteins onto gel was confirmed by immunodetection of beta-actin as exemplified in **Fig. 6.**

Fig. 6. Effect of atorvastatin on endoglin protein expression. Atorvastatin treatment increased endoglin expression. Values are means ± SEMs of six measurements; representative immunoblots are given below; *** $p < 0.001$ vs. control.

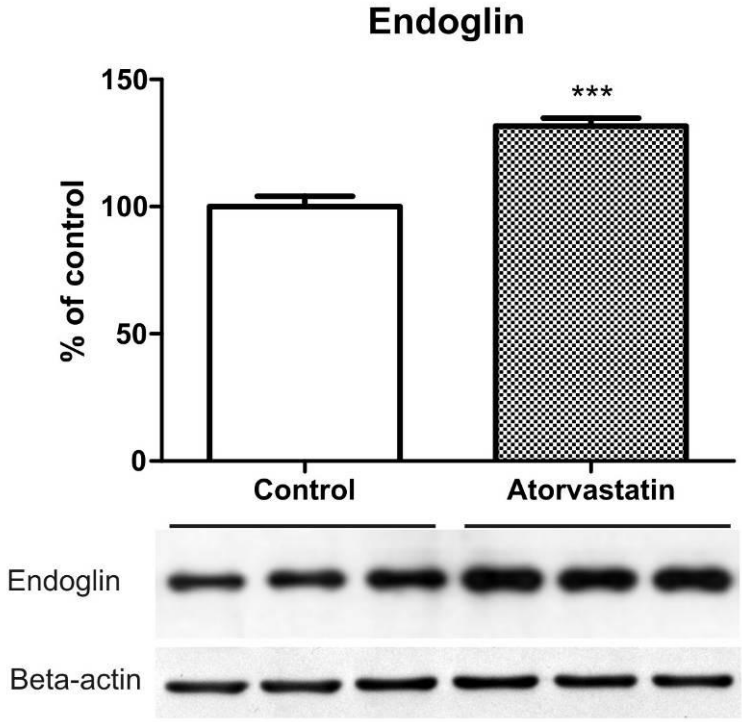


Fig. 7. Effect of atorvastatin on SMAD2 protein expression. Atorvastatin treatment increased SMAD2 expression. Values are means ± SEMs of six measurements; representative immunoblots are given below; *** $p < 0.001$ vs. control.

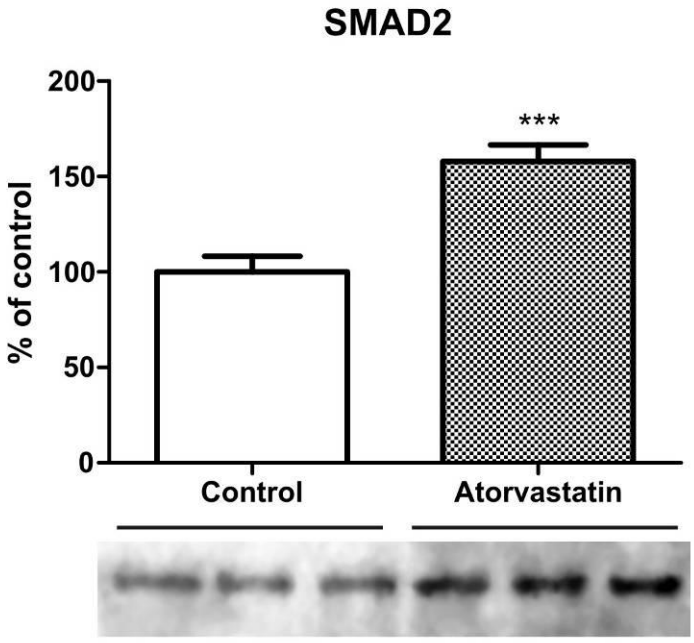


Fig. 8. Effect of atorvastatin on P-SMAD2/3 protein expression. Atorvastatin treatment increased P-SMAD2/3 expression. Values are means ± SEMs of six measurements; representative immunoblots are given below; *** $p < 0.001$ vs. control.

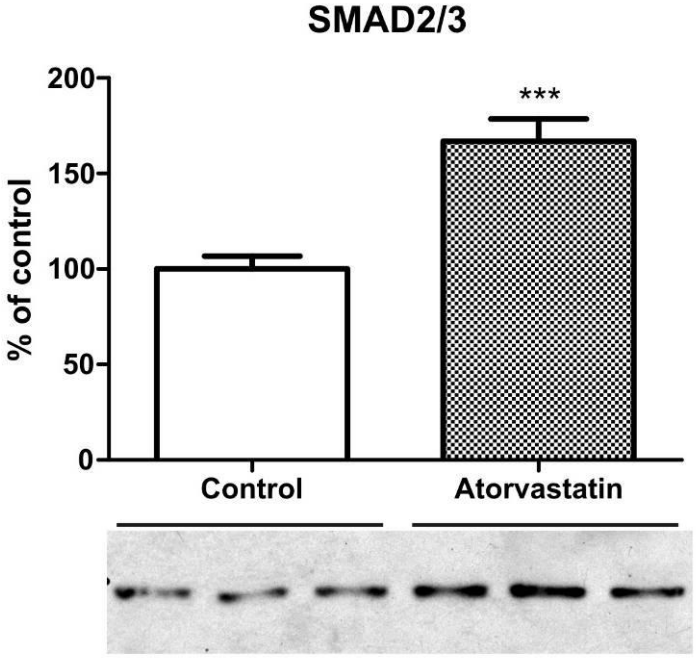
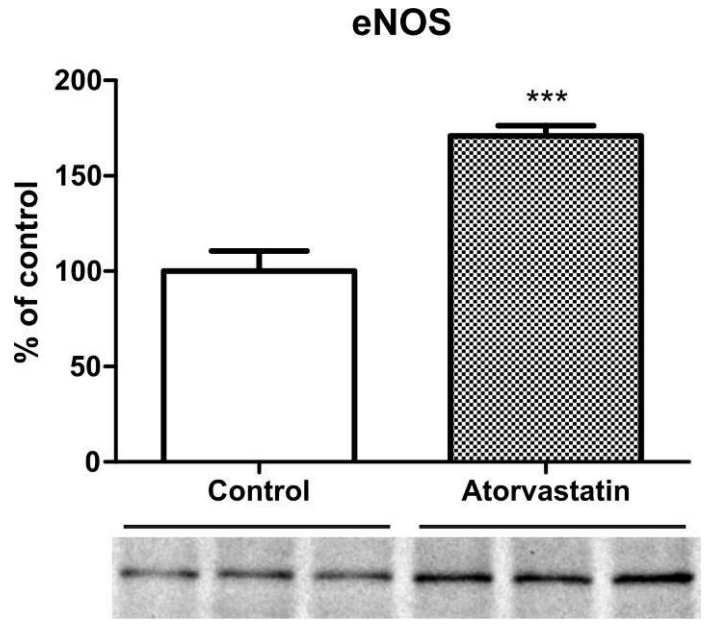


Fig. 9. Effect of atorvastatin on eNOS protein expression. Atorvastatin treatment increased eNOS expression. Values are means ± SEMs of six measurements; representative immunoblots are given below; *** $p < 0.001$ vs. control.



Discussion

Endoglin (or CD105) is a homodimeric membrane glycoprotein that in association with TGF-β receptors binds TGF-β1 and - β3 isoforms in human endothelial cells (Zhang 1996). In addition, increased endoglin expression is observed in endothelial cells (ECs) of microvessels from pathological skin lesions and in the neovessels of tumors, suggesting a role for endoglin during endothelial cell proliferation (Letamendia 1998).

The role of endoglin in atherogenesis was also studied recently. Endoglin was expressed at low levels in normal porcine and human coronary arteries and overexpressed in diseased arteries not only endothelial cells and fibroblasts but transiently in smooth muscle cells and macrophages (Conley 2000, Ma 2000, Piao 2006). Moreover, its expression was detected universally in microvessels within the atheroma suggesting the role in plaque angiogenesis (Li 2006).

On the contrary, in this study we found the expression of endoglin predominantly in endothelium covering the atherosclerotic lesion, aortic valves, outside the lesion and in the capillaries of surrounding myocardium in advanced atherosclerotic lesions in ApoE/LDLR double knockout mice. We found only a weak expression of endoglin inside atherosclerotic plaque and almost no expression by smooth muscle cells in vessel media. These results are consistent with our previous studies in non-atherosclerotic vessels in normo- and hypercholesterolemic mice (Nachtigal 2007, Pospisilova 2006) suggesting that endoglin is expressed predominantly by vessel endothelium in mice. This discrepancy with previous mentioned studies in humans might reflect the differences between human and mice atherogenesis.

Furthermore, it has been shown that endoglin regulates nitric oxide-dependent vasodilatation, as well as eNOS expression and activity (Jerkic 2004, Toporsian 2005) In this study, fluorescence immunohistochemistry revealed co-localization of endoglin and eNOS in aortic vessel endothelium covering the atherosclerotic plaque only, suggesting the possible role of endoglin in endothelium during atherogenesis. Moreover, recently Santibanez et al demonstrated that endoglin enhances eNOS expression by potentiating SMAD2 protein levels *in vitro* suggesting the role of endoglin in the regulation of eNOS expression (Santibanez 2007).

Endoglin forms complexes with TβRI and TβRII, and has been postulated to facilitate binding of TGF-β1 to these signaling receptors suggesting that endoglin affects TGF-β signaling events (Bobik 2006). However, increasing evidence also indicates that endoglin may have functions independent of TGF-β1. For example, only about 1% of the endoglin molecules on endothelial cells bind TGF-β, suggesting that endoglin has another, undefined physiological ligand (Conley 2000). Furthermore it was demonstrated that endoglin upregulates eNOS expression at the transcriptional level, both in the absence and in the presence of exogenous TGF-β in endothelial cells (Chen 2008, Chen 2007, Mallat 2002).

We therefore focused on the endoglin relationship with SMAD proteins in our experiment. It was demonstrated that SMAD2 inhibits proinflammatory adhesion molecules such as E-Selectin and at the same time induce eNOS expression (Bobik 2006, Feinberg 2005, Saura 2002).

To the best of our knowledge, there are no *in vivo* studies showing that the above-mentioned proteins are at least expressed simultaneously in the same cells in the atherosclerotic lesions.

In this study, we demonstrated strong co-localization of endoglin with SMAD2 and phosphorylated SMAD2/3 in aortic vessel endothelium. Despite the fact that endoglin, SMAD2 and phosphorylated SMAD2/3 staining was detected even inside the

atherosclerotic lesion no co-localization of these proteins was found. Thus, here we demonstrate for the first time that endoglin, SMAD2, phosphorylated SMAD2/3 and eNOS are expressed together only by endothelial cells *in vivo* in advanced atherosclerotic lesions in mice suggesting their possible role in vessel endothelium homeostasis and atherogenesis.

We also hypothesized whether endoglin together with SMADs and eNOS could be affected by the "antiatherogenic" drug treatment *in vivo*. We therefore used atorvastatin to assess its effects on endoglin, SMAD2, phosphorylated SMAD2/3, and eNOS expression in mouse aorta. In our recent paper we demonstrated, strong hypolipidemic and anti-inflammatory effects of atorvastatin represented by decreased MCP-1 levels in blood, and decreased VCAM-1 and ICAM-1 expression in the vessel wall in ApoE/LDLR double knockout mice (Nachtigal 2008). In this study with same experimental design atorvastatin treatment significantly increased the expression of endoglin, SMAD2, phosphorylated SMAD2/3 and eNOS in aorta. Since it was demonstrated that endoglin enhances the SMAD2 signaling pathway (Santibanez 2007) and inhibits the SMAD3 signaling pathway, (Blanco 2005, Lebrin 2005) we propose that increased expression of phosphorylated SMAD2/3 proteins mostly exhibit an increase of phosphorylated SMAD2 protein. Thus, atorvastatin treatment resulted in strong hypolipidemic and anti-inflammatory effects (Nachtigal 2008) with concurrent increase of endoglin, SMAD2, phosphorylated SMAD2/3 and eNOS expression suggesting positive effect of statin treatment on aortic vessel endothelium in ApoE/LDLR double knockout mice.

On the contrary, in our previous study we found that atorvastatin decreased endoglin expression by both hypolipidemic and pleiotropic effects (Nachtigal 2006, Nachtigal 2007). However, there are some substantial differences when we compare results of this study with previous ones. Firstly, previous study was made in different mouse strains, apoE-deficient mice (Nachtigal 2006) and in C57BL/6J mice (Nachtigal 2007). Secondly, in previous experiments we did not detect any atherosclerotic lesions in these mice and quantified the expression of endoglin in intact non-atherosclerotic endothelium. We propose that the expression of endoglin might be regulated differently in intact non-atherosclerotic vessel when compared with vessels with advanced atherosclerotic lesions. Moreover, the difference of endoglin expression and quantity here and in previous studies could be also related to the lipid metabolism. In the first place cholesterol levels in this study with apoE/LDL receptor deficient mice were markedly higher when compared with previous experiments with C57BL/6J and apoE-deficient mice. Moreover atorvastatin effects on endoglin expression could also be related to the presence of LDL receptor which should be markedly increased after statin treatment. In this study mice do not have LDL receptor which means that atorvastatin effects must be related to the different mechanism. However we believe that the precise relationship between cholesterol levels and endoglin expression must be elucidated in prospective *in vitro* study in endothelial cells (HUVEC).

The increase of eNOS expression after statin treatment was previously demonstrated and related to a decrease in LDL and/or through statins inhibition of Rho geranylgeranylation through statins (Blair 1999, Laufs 1998). It was also suggested that endoglin might be proatherogenic marker participating in the development and progression of atherosclerosis (Conley 2000, Ma 2000, Piao 2006). On the contrary we might propose that the increase of eNOS expression in this study after atorvastatin treatment was related to the hypolipidemic effect of atorvastatin shown previously (Nachtigal 2008), which likely caused upregulation of endoglin and SMAD2 expression in the vessel wall. This hypothesis is partially consistent with very recent *in vitro* data of Chen et al. who clearly demonstrated that cholesterol suppresses and statins enhance

SMAD2 phosphorylation (Chen 2008, Chen 2007). However, *in vitro* mechanistic study elucidating the influence of cholesterol and/or statins on endoglin expression in endothelial cells is needed.

In conclusion, we demonstrate here for the first time that endoglin is co-expressed with SMAD2, phosphorylated SMAD2/3 and eNOS only in aortic endothelium *in vivo*, in ApoE/LDLR double knockout mice. In addition, we have shown that statin treatment significantly induced expression of all above-mentioned proteins in the vessel wall. Therefore, these findings suggest, that endoglin might be interesting marker of endothelial dysfunction and/or atherogenesis which is upregulated by statins implicating potential beneficial role of endoglin molecule in atherosclerosis.

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VIII.

SOUHRN / SUMMARY

Ateroskleróza je jednou z hlavních příčin kardiovaskulárních onemocnění. Jde o chronické zánětlivé onemocnění, charakterizované endoteliální dysfunkcí s následným hromaděním lipidů, leukocytů, hladkých svalových buněk a extracelulární matrix v intimě cév, což má za následek zužování cévního lumen s následnou redukcí až obstrukcí cévního průtoku. Na počátku aterogenního procesu je změna funkce endotelu tzv. endoteliální dysfunkce. Dochází k expresi adhezních molekul a ke zvýšené propustnosti endotelu pro monocyty, T lymfocyty a také lipoproteinové částice. Spouští se bludný kruh aterosklerotického procesu.

Mezi nejvýznamnější adhezní molekuly, které se uplatňují na počátku aterogeneze patří zejména zástupci imunoglobulinové skupiny a to VCAM-1 (vascular cell adhesion molekule-1), ICAM-1 (intracellular cell adhesion molekule-1) a PECAM-1 (platelet-endothelial cell adhesion molekule-1). VCAM-1 a ICAM-1 jsou transmembránové glykoproteiny, které se podílejí na stabilizaci vazby leukocytů k endotelu a podílejí se na jejich diapedezi. Exprese těchto adhezních molekul je ovlivňována řadou faktorů, které se uplatňují i v patogenezi aterosklerózy.

V rámci této disertační práce jsme se zaměřili na endoteliální expresi adhezních molekul VCAM-1 a ICAM-1 a její změny po podávání atorvastatinu a dalších látek u několika myších modelů aterosklerózy.

Porovnávali jsme účinky krátkodobého podávání simvastatinu, atorvastatinu a MDOC™ na endoteliální expresi VCAM-1 a ICAM-1 u apoE-deficietních myší. Myši byly krmeny standardní dietou s přídavkem simvastatinu, atorvastatinu a MDOC™ po dobu 4 týdnů. Byla provedena biochemická analýza a imunohistochemická detekce exprese VCAM-1 a ICAM-1, která byla kvantifikována pomocí stereologických metod. Tato studie přinesla zejména nové poznatky o látce MDOC™. Prokázali jsme, že má potenciální hypolipidemické a protizánětlivé účinky. Také jsme potvrdili protizánětlivé účinky atorvastatinu nezávislé na jeho hypolipidemickém účinku.

Dále jsme porovnávali vliv 8 týdenního podávání atorvastatinu na endoteliální expresi těchto dvou molekul u apoE-deficientních myší a C57BL/6J myší, krmených standardní nebo aterogenní dietou. Prokázali jsme, že u myší C57BL/6J exprese adhezních molekul nesouvisí s hladinou cholesterolu v krvi. Potvrdili jsme, že statiny působí protizánětlivě i jiným mechanismem než

snižováním hladiny lipidů v krvi, neboť 8 týdenní podávání statinů snížilo expresi adhezních molekul u myší C57BL/6J krmených standardní dietou a to bez ovlivnění hladin lipidů v séru.

V další studii jsme sledovali účinky atorvastatinu u relativně nového myšího modelu ateroskleróz - apoE/LDLr deficientních myší. Sledovali jsme vliv na hladiny lipidů v séru, vliv na hladinu monocytárního chemotaktického proteinu (MCP-1), ovlivnění velikosti aterosklerotických lézí a také ovlivnění exprese adhezních molekul VCAM-1 a ICAM-1. Prokázali jsme hypolipidemické a protizánětlivé účinky statinů i u tohoto myšího modelu. Předpokládáme, že právě tento model by mohl být vhodným modelem pro studium účinku statinů a dalších látek, které by se daly využít v kombinační léčbě právě se statiny.

Druhou částí této disertační práce bylo určení lokalizace exprese endoglinu v cévě postižené aterosklerózou a ovlivnění této exprese hladinou cholesterolu v krvi a podáváním statinu.

Endoglin (CD 105) je komponenta TGF-β-receptorového komplexu, který zprostředkovává buněčnou odpověď na TGF-β 1. Je přítomný v endotelových buňkách, účastní se angiogeneze, kardiovaskulárního vývoje a cévní homeostázy. Jeho zvýšená exprese byla sledována na aktivovaných makrofázích, a také na endoteliálních buňkách ve tkáních, ve kterých probíhá angiogeneze, jako jsou hojící se rány, infarkty a celá řada nádorů. Zvýšená exprese je dokázána v hladkých svalových buňkách cév během zánětu, poranění a v aterosklerotických lézích.

V našich studiích na myších modelech ateroskleróz jsme prokázali expresi endoglinu zejména v oblasti aortálního oblouku, a to hlavně v buňkách aktivovaného endotelu, který pokrývá aterosklerotické léze a chlopně. Mimo to byl endoglin velmi silně byl exprimován buňkami kapilár v okolním myokardu.

Sledovali jsme vliv hladiny cholesterolu v krvi na endoteliální expresi endoglinu a také změny exprese po podávání atorvastatinu u myší kmene C57BL/6J. Hypercholesterolémie byla u myší navozena aterogenní dietou. U myší nedošlo k rozvoji aterosklerotických lézí, šlo tedy pouze o stadium endoteliální dysfunkce. Prokázali jsme, že exprese endoglinu souvisí s hladinou cholesterolu v krvi a je snížena po podávání statinů. Je tedy velmi pravděpodobné, že endoglin má svoji úlohu v procesu aterogeneze.

Pro potvrzení této myšlenky jsme v další studii sledovali expresi endoglinu a její ovlivnění u jiného myšího modelu aterosklerózy - apoE-deficientních myší. Myši byly krmené standardní dietou a atorvastatin byl podáván po dobu 4 a 8 týdnů. Ani u těchto myší nedošlo k rozvoji aterosklerotických lézí. Tato studie přinesla další informace o expresi endoglinu během časné fáze aterogeneze. Potvrdili jsme, že zvýšená hladina lipidů v krvi zvyšuje expresi endoglinu a navíc se ukázalo, že atorvastatin snižuje jeho expresi nezávisle na svém hypolipidemickém účinku. Na základě výsledků těchto studií usuzujeme, že endoglin je možným markerem endoteliální dysfunkce.

V naší poslední studii, jsme se zaměřili na expresi endoglinu a expresi eNOS, p-Smad2/3 a Smad2 proteinu. V pokusech *in vitro* je prokázáno, že endoglin podporuje vazodilataci, zvyšuje expresi eNOS a to ovlivněním hladiny proteinu Smad2. My jsme sledovali expresi endoglinu, Smad2, p-Smad2/3 a eNOS *in vivo*, v cévách apoE/LDLr deficientních myší, které byly krmené aterogenní dietou. Současně jsme sledovali také vliv podávání statinu na expresi těchto markerů. U myší byly detekované výrazné aterosklerotické léze. Pomocí fluorescenční imunohistochemie jsme vyhodnotili kolokalizaci exprese endoglinu, Smad2, p-Smad2/3 a eNOS, která byla výrazná zejména na endotelu pokrývajícím aterosklerotické léze. Western blot analýza prokázala výrazně vyšší expresi endoglinu, Smad2, pSmad2/3 i eNOS v aortě po 8-týdením podávání atorvastatinu ve srovnání s neléčenou skupinou.

Při porovnání výsledků našich dosavadních studií můžeme říci, že endoglin je potenciální znak endoteliální dysfunkce a aterogeneze a může mít pravděpodobně antiaterogení vlastnosti, ovšem jeho úloha je pravděpodobně závislá na stupni rozvoje aterosklerózy. Nicméně další studie by jeho úlohu v aterogenezi měly objasnit.

Atherosclerosis is one of the major causes of cardiovascular morbidity. This chronic inflammatory disease is characterized by endothelial dysfunction with accumulation of lipids, leukocytes, smooth muscle cells and extracellular matrix within the vessel intima. This process results in reducing of the vessel lumen that can lead to the obstruction of vessel blood flow. The initiation of atherogenic process is characterized by the alteration of endothelial function which is so-called endothelial dysfunction. The endothelial dysfunction is characterized by the expression of cell adhesion molecules and increased endothelial permeability for monocytes, T-cells and lipoproteins. This is beginning of „vicious"circle of atherosclerosis.

On of the most important cell adhesion molecules participating in the beginning of atherogenesis are members of the immunoglobulin superfamily, VCAM-1 (vascular cell adhesion molekule-1), ICAM-1 (intracellular cell adhesion molekule-1) and PECAM-1 (platelet-endothelial cell adhesion molekule-1).VCAM-1 and ICAM-1 are transmembrane glycoproteins participacing predominantly in the stabilization leukocyte of interaction with endothelium and transmigration of leukocytes into vessel intima.

In this dissertation thesis, the first studies were focused on the endothelial expression of VCAM-1 and ICAM-1 in mouse aorta. Moreover the effects of statin treatment on the expression of these molecules were studied in several mouse models of atherosclerosis.

The changes of endothelial expression of VCAM-1 and ICAM-1 in the vessel wall after the short-term administration of simvastatin, atorvastatin, and micro dispersed derivates of oxidised celulose (MDOC™) in apoE-deficient mice were studied. Mice received normal chow diet or diet containing simvastatin or atorvastatin 10 mg/kg/day or MDOC™ 50 mg/kg/day. Biochemical analysis and stereological analysis of the immunohistochemical staining of VCAM-1 and ICAM-1 were performed. Atorvastatin treatment resulted in reduced expression of both adhesion molecules suggesting that atorvastatin has anti-inflammatory effects independent of hypolipidemic effects.

Furthermore, we compared the effect of 8-week atorvastatin treatment on both lipid parameters and VCAM-1 and ICAM-1 expression in apoE-deficient or wild type C57BL/6J mice that were fed with either chow or atherogenic diet. We

demonstrated that endothelial expression of both VCAM-1 and ICAM-1 does not correlate with cholesterol levels in blood. Moreover, we showed that 8-week administration of atorvastatin decreased endothelial expression of these adhesion molecules in C57BL/6J mice fed by chow diet beyond its lipid lowering effect.

We focused on atorvastatin effects in new mouse model of atherosclerosis – apoE/LDLr deficient mice in other study. Atorvastatin significantly decreased cholesterol levels, monocyte chemotactic protein-1 (MCP-1) levels in blood, and expression of cell adhesion molecules VCAM-1 and ICAM-1 in the vessel wall. We demonstrated strong hypolipidemic and anti-inflammatory effects of atorvastatin in this mouse model. We propose that this model might be a good animal model for the study of effects of statins and other substances that could be used in combination treatment with statins.

The second part of this dissertation was focused on endoglin role in atherogenesis. We studied endoglin expression in the vessel wall and possible effects of atorvastatin treatment on this expression.

Endoglin (CD 105) is a part of the transforming growth factor-β (TGF-β) receptor complex that affects TGF-β1 signaling. The major sources of endoglin are vascular endothelial cells. Endoglin participates in angiogenesis, cardiovascular development and vascular homeostasis. Moreover endoglin is highly expressed in activated macrophages, in tissues undergoing angiogenesis such as healing wounds, infarcts and in wide range of tumors. In addition its enhanced expression was documented in smooth muscle cells of vessels during inflammation, injury and in human atherosclerotic plaques.

In the first study we wanted to evaluate whether endoglin is expressed in normocholesterolemic and hypercholesterolemic C57BL/6J mice as well as whether it is affected by atorvastatin treatment in these mice. Biochemical analysis of blood samples revealed that administration of atherogenic diet significantly increased levels of total cholesterol, VLDL, LDL and decreased levels of HDL. Atorvastatin treatment resulted in a significant decrease of total cholesterol and VLDL only in mice fed by atherogenic diet. Quantitative stereological analysis revealed that atorvastatin significantly decreased endothelial expression of endoglin in C57BL/6J mice fed with atherogenic diet only. In conclusion we demonstrated that endothelial expression of endoglin is upregulated by

hypercholesterolemia and decreased by hypolipidemic effect of atorvastatin in C57BL/6J mice suggesting that endoglin expression could be involved in atherogenesis.

In the second study we hypothesized whether endothelial expression of endoglin is changed in hypercholesterolemia as well as whether its expression is affected by atorvastatin treatment in apoE-deficient mice. This study demonstrated that endoglin was expressed by aortic endothelium showing similar staining patterns like other markers involved in the process of atherosclerosis. In addition, we showed that endoglin expression in endothelium could be affected by the administration of atorvastatin beyond its lipid lowering effects in apoE-deficient mice

On the basis of results of these studies, we concluded endoglin may be possible marker of endothelial dysfunction.

The last study we stepped forward to elucidate whether endoglin is co-expressed with SMAD2, phosphorylated SMAD2/3 proteins and eNOS in vivo in atherosclerotic lesions in ApoE/LDLR double knockout mice. In addition, we sought whether endoglin expression as well as the expression of SMAD2, phosphorylated SMAD2/3 and eNOS is affected by atorvastatin treatment. Immunohistochemical and western blot analysis of endoglin, SMAD2, phosphorylated SMAD2/3 and eNOS expressions in aorta were performed. The biochemical analysis showed that administration of atorvastatin significantly decreased level of total cholesterol, VLDL, LDL, TAG, and significantly increased level of HDL cholesterol. Fluorescence immunohistochemistry showed endoglin co-expression with SMAD2, phosphorylated SMAD2/3 and eNOS in aortic endothelium covering atherosclerotic lesions in both control and atorvastatin treated mice. Western blot analysis demonstrated that atorvastatin significantly increased expression of endoglin, SMAD2, phosphorylated SMAD2/3, and eNOS in mice aorta.

In conclusion, these findings suggest, that endoglin might be interesting marker of endothelial dysfunction and/or atherogenesis which is upregulated by statins implicating potential beneficial role of endoglin and its pathway in atherosclerosis.

IX.

SEZNAM PUBLIKOVANÝCH PRACÍ

PŮVODNÍ PRÁCE PUBLIKOVANÉ V ODBORNÝCH ČASOPISECH

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