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Molekulární podklady endotelové dysfunkce: genetické varianty endotelové syntázy oxidu dusnatého a hemoxygenázy 1

Molecular basis of endothelial dysfunction: endothelial nitric oxide synthase and heme oxygenase 1 genetic variations

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Abstrakt

Endoteliální dysfunkce je patologický stav charakterizovaný narušenou rovnováhou mezi vazodilatačními a antitrombotickými mediátory a vazokonstriktivními a protrombotickými mediátory produkovanými cévním endotelem. Řada faktorů vede k narušení produkce nebo zvýšené konsumpci oxidu dusnatého (NO), produkovaného syntázami oxidu dusnatého (NOS), který je klíčovým mediátorem cévní homeostázy. Endoteliální dysfunkce představuje jeden z počátečních kroků v rozvoji aterosklerózy, chronického zánětlivého onemocnění cévní stěny. Inducibilní enzym hem oxygenáza 1 (HO-1) představuje jeden z hlavních buněčných obranných mechanismů proti zvýšenému oxidativnímu stresu a snížené dostupnosti NO, které jsou průvodními jevy endoteliální dysfunkce a aterosklerózy. Studovali jsme genetické determinanty endoteliální dysfunkce a aterosklerózy pomocí hodnocení vlivu G894T polymorfizmu endotelové NOS (eNOS) a (GT)n polymorfizmu promotoru HO-1 na rozsah a rizikový profil koronární aterosklerózy a jejich vývoje během hypolipidemické léčby. Dále jsme hledali genetické varianty v exonech 25 a 26 genu eNOS kódujících C-terminální část proteinu, která je považovaná za klíčovou pro správnou funkci enzymu a 3´ nepřepisovanou oblast klíčovou pro stabilitu eNOS mRNA. Nenalezli jsme asociaci eNOS G894T polymorfizmu s rozsahem a rizikovým profilem koronární aterosklerózy, nicméně jsme pozorovali jeho vliv na změny složení aterosklerotických plátů během hypolipidemické léčby. Objevili novou variantu (G3911A) v nekódující mRNA exonu 26, nález zasluhující další výzkum pro jeho možný vliv na posttranskripční regulaci eNOS. Rizikový polymorfizmus HO-1 promotoru pozitivně koreloval s rozsahem koronární aterosklerózy a rizikovými charakteristikami koronárních plátů.

Klíčová slova: endoteliální dysfunkce, ateroskleróza, oxid dusnatý, endotelová syntáza oxidu dusnatého, hem oxygenáza 1, polymorfizmus

Abstract

Endothelial dysfunction is a pathologic state characterized by an altered equilibrium among vasodilatory and antithrombotic mediators and vasoconstrictive and prothrombotic mediators produced by the vascular endothelium. Multiple factors induce impaired production or increased consumption nitric oxide (NO), the key mediator of vascular homeostasis, produced by the nitric oxide synthase enzymes (NOS). Endothelial dysfunction represents one of the initial steps in the development of atherosclerosis, a chronic inflammatory disease of the vascular wall. The inducible enzyme heme oxygenase 1 (HO-1) represents one of the main

cellular defense mechanisms against increased oxidative stress and decreased NO bioavailability accompanying endothelial dysfunction and atherosclerosis. We studied the genetic determinants of endothelial dysfunction and atherosclerosis by evaluating the association of the G894T endothelial NOS (eNOS) polymorphism and the HO-1 (GT)n promoter polymorphism with coronary artery atherosclerosis severity and risk profile and their evolution during hypolipidaemic treatment. In addition, we searched for genetic variations in exons 25 and 26 of eNOS gene, encoding the C-terminal part of the protein, deemed crucial for proper enzyme function and the 3′- untranslated region crucial for eNOS mRNA stability. We did not find an association of the eNOS G894T polymorphism with the extent and risk profile of coronary atherosclerosis, nevertheless we observed its association with atherosclerotic plaque composition changes during hypolipidaemic therapy. We described a novel variant (G3911A) in the noncoding mRNA of exon 26, a finding warranting further research of its possible impact on eNOS posttranscriptional regulation. The risk HO-1 promoter polymorphism was found to positively correlate with coronary artery atherosclerotic burden and high-risk coronary plaque features.
Key words: endothelial dysfunction, atherosclerosis, nitric oxide, endothelial nitric oxide synthase, heme oxygenase 1, polymorphism

1. Introduction

Endothelial dysfunction is a pathologic state characterized by an imbalance among vasodilatory and antithrombotic mediators and vasoconstrictive and prothrombotic mediators produced by the vascular endothelium leading to decreased anticoagulation properties of the endothelium, increased expression of adhesive molecules and increased cytokine and reactive oxygen species production (ROS) by the endothelia (Ignarro et al., 1999). Multiple mechanisms lead to impaired production or dysregulation of pivotal mediators of vascular homeostasis, chiefly nitric oxide (NO), a gaseous molecule produced by the nitric oxide synthase (NOS) enzymes. Endothelial dysfunction, characterized by a loss of NO bioavailability, is associated with all major cardiovascular risk factors and represents a mechanism leading to the development and progression of atherosclerosis, a chronic inflammatory disease of the vessel wall resulting from an excessive inflammatory fibroproliferative response to various forms of insult to the endothelium and vascular smooth muscle cells (VSMC) characterized by accumulation of lipids and fibrous elements (Araujo et al., 2012; Li and Forstermann, 2013). The heme oxygenase (HO) family of enzymes catalyzes

the degradation of heme to biliverdin, carbon monoxide (CO) and free iron (Fe^{2+}). The inducible form, HO-1 is designated as one of the main cellular defense mechanisms against increased oxidative stress and decreased NO bioavailability accompanying endothelial dysfunction and atherosclerosis (Abraham and Kappas, 2008).

The nitric oxide synthase family comprises of three enzyme isoforms, the neuronal (nNOS), the inducible (iNOS), and endothelial NOS (eNOS). The eNOS gene consists of 26 exons, spans 21 kb and is located on chromosome 7 (7q35-36) encoding a 1203-aminoacid 133-kDa protein. The eNOS gene is predominantly expressed in the endothelium and cardiomyocytes. ENOS derived NO is one of the major determinants of vascular tone, inhibitor of vascular adhesion molecule production and vascular smooth muscle cell (VSMC) proliferation. All NOS isoforms share a similar structure, a N-terminal oxygenase domain connected via a calmodulin-binding amino acid sequence to a reductase domain containing two flavins FMN (flavin mononucleotide) and FAD (flavin adenine dinucleotide) and a NAPDH (nicotinamide adenine dinucleotide phosphate) amino acid binding sequence. The oxygenase domain represents the catalytic part of the enzyme. The enzymes catalyze a reaction in which L-arginine is oxidized to NO and L-citrullin. NADPH, molecular oxygen and the cofactors Zn^{2+} and tetrahydrobiopterin (BH_4) are essential for proper enzyme function. NOS isoforms differ in catalytic activity, regulation and cellular localization (Roman et al., 2002). The C-terminal parts of the reductase domain of NOS determine the rate of reduction equivalent transfer to the heme part of the protein. These C-termini slow electron transfer between the two flavins, enabling tight electron coupling, thereby suppressing superoxide production (Jáchymová et al., 2005). In endothelial dysfunction, electron coupling is altered, giving rise to an increased amount of superoxide radicals. Upon stimulation by various agonist stimuli, the eNOS produces NO, which activates the primary NO receptor, the soluble guanylate cyclase (sGC). Furthermore, NO initiates nitrosation reactions, i.e. S-nitrosylation. Upon activation, the sGC produces cGMP (cyclic guanosine monophosphate), which functions as a second messenger to stimulate protein kinase G (PKG)-dependent activation of myosin light chain phosphatase (MLCP), thereby promoting VSMC relaxation, leading to vasodilatation, and inhibition of thrombocyte reactivity. Alternatively, cGMP may modulate VSMC relaxation by decreasing intracellular levels of Ca^{2+} via several mechanisms. S-nitrosylation contributes to the anti-proliferative actions of NO and ion channel modulation exerted by NO in the vascular wall. Moreover, NO decreases endothelial expression of adhesion molecules implicated in cellular migration, proliferation, atherogenesis, and thrombosis and inhibits leukocyte adhesion to the vessel wall (Maron and Michel 2012;

Forstermann and Sessa, 2012). The bioactivity of eNOS derived NO is fundamentally determined by the balance between the synthesis and degradation rate of the molecule. NO bioavailability is regulated at the levels of eNOS gene expression, the eNOS enzymatic activity and lastly at the level of NO degradation. The enzymatic activity of eNOS is highly regulated by multiple mechanisms including eNOS lipidation, phosphorylation, O-glycosylation, *S*-nitrosylation, S-glutathionylation and direct protein-protein interactions. Furthermore, substrate and cofactor availability, as well as endogenous inhibitors of eNOS, all contribute to the "fine-tuning" of NO production. Dysregulation of any of these mechanisms can cause endothelial dysfunction and ensuing atherosclerosis.

Genetic variants of eNOS influence eNOS activity at several levels. Two functionally relevant polymorphisms in the eNOS affecting eNOS transcriptional activity have been identified. The T(-786)C variant located in the gene promoter results in significantly reduced transcriptional activity, while the 4 b/a polymorphism determines the number of 27-nt repeats in intron 4, giving rise to microRNAs (miRs), shown to significantly suppress transcriptional activity (Nakayama et al., 1999; Zhang et al., 2005). Differently, a single-nucleotide polymorphism (SNP) in exon 7 of the eNOS gene, the G894T polymorphism, coding for a substitution of Glu by Asp at position 298 (Glu298Asp variant) in the oxygenase domain is associated with decreased NO production in endothelial cells under basal conditions and in response to shear stress. This alteration seems to affect eNOS protein binding to caveolin-1 (Cav-1), thus resulting in its reduced association with the caveolar fraction (Joshi et al., 2007). Even though a full explanation of the mechanisms by which the Asp variant influences eNOS enzyme activity *in vivo* is still lacking, fairly robust data has been accumulated over the past years in favor of the functional significance of this common variant, including an enhanced systemic pressor response to phenylephrine, lower basal flow with a preserved response to adenosine in coronary arteries, vasospastic angina pectoris, essential hypertension resistant to conventional antihypertensive therapy and increased risk of carotid and coronary artery atherosclerosis (Balligand et al., 2009; Li et al., 2010).

All major cardiovascular risk factors lead to a reduction in NO bioavailability, resulting from decreased NO production and augmented NO inactivation by ROS. Under certain conditions, eNOS can become proatherogenic, i.e., uncoupling of eNOS, characterized by a diversion of electron flow from the enzyme substrate L-arginine to molecular oxygen resulting in the production of the superoxide radical instead of NO. Several mechanisms are implicated in eNOS uncoupling, including BH_4 deficiency, depletion of L-arginine, asymmetric dimethylarginine (ADMA) accumulation and S-glutathionylation (Li and

Förstermann, 2013). Currently, eNOS uncoupling is attributed a key role in atherogenesis. Current evidence suggests that atherosclerotic endothelial dysfunction is primarily caused by a dysregulation of eNOS enzymatic activity and NO inactivation through increased oxidative stress.

The heme oxygenase (HO) protein family comprises of three isoforms, HO-1, 2, 3. These enzymes catalyze the first and rate-limiting step in the degradation of the protoporphyrin ring of heme, yielding equimolar quantities of biliverdin IXa, CO, and Fe^{2+}. Biliverdin is subsequently rapidly converted to bilirubin by biliverdin reductase. The HO-1 is an enzyme inducible by a variety of stimuli, many of these leading to ROS generation, including heme, endotoxin, heavy metals, oxidants, hypoxia and hyperoxia, cytokines (IL-10, 13,18), growth factors (PDGF, VEGF), oxidized LDL and UV light. Substantial evidence has been presented that biliverdin and bilirubin represent a cellular defense strategy in response to oxidative stress, acting to prevent oxidant-mediated cell death. High-normal serum levels of bilirubin are inversely related to atherogenesis. Atheroprotective actions of bilirubin include inhibition of LDL oxidation, scavenging of ROS and reactive nitrogen species (RNS), attenuation of NADPH oxidase activity, resulting in decreased superoxide production and inhibition of PKC activity. In addition, bilirubin attenuates pro-inflammatory responses of the endothelium. CO has a physiological role in the regulation of vascular tone similar to that of NO, in part acting through cGMP. In addition, CO exerts numerous cGMP-independent vasculoprotective actions. In summary, bilirubin and CO seem to provide protection against the development of endothelial dysfunction and atherosclerosis (Morita, 2005; Abraham and Kappas, 2008).

HO-1 activity is principally regulated at the level of gene expression. HO-1 transcription is determined by responsive elements localized in the promoter 5´-flanking region of the HO-1 gene. Inducers of HO-1 activate different protein phosphorylation-dependent signaling cascades that ultimately converge to modulate a complex system of transcriptional regulators. Studies of the HO-1 promoter region have identified numerous transcriptional factors as well as multiple response elements for transcription factors (Morita, 2005; Ryter et al., 2006). The concept of a causal relationship of HO-1 to cardiovascular diseases has been suggested by studies assessing polymorphisms in the 5´-flanking sequence of the HO-1 gene. Undoubtedly, the most studied one so far is the (GT)n dinucleotide-length polymorphism. The number of glutathione thymidine (GT) dinucleotide repeats in the promoter of the HO-1 gene modulates the level of gene transcription. The long alleles lead to decreased HO-1 inducibility, whereas the short alleles demonstrate adequate HO-1 expression

upon stimulation (Exner et al., 2001; Morita et al. 2005). Genetic association studies have reported the relevance of this polymorphism to many disease states caused by increased oxidative stress (Exner et al., 2001). Importantly, HO-1 deficiency has been described in humans, leading to accelerated atherosclerosis, suggesting that HO-1 plays a crucial role in vasculoprotection against oxidative insults (Yachie et al., 1999). Furthermore, numerous findings provide strong evidence for a beneficial effect of HO-1 in experimental atherosclerosis. Many researchers have therefore suggested that up-regulation of HO-1 may represent a protective mechanism counteracting endothelial dysfunction and atherosclerosis and thus imply HO-1 as a potential novel therapeutic target.

2. Aims and hypotheses

The aim of our work was to examine the genetic determinants of endothelial dysfunction and atherosclerosis by assessing variations in the eNOS a HO-1 genes.

2.1. We sought to identify whether genetic variations in the C-terminal part of eNOS, deemed crucial for proper enzyme function, are present in patients with coronary and/or peripheral artery disease.
We tested the hypothesis that genetic variants in the C-terminal part of the eNOS gene represent a novel genetic factor contributing to endothelial dysfunction and atherosclerosis development.

2.2. We sought to elucidate the influence of the eNOS Glu298Asp polymorphism and of the HO-1 promoter (GT)n polymorphism on the extent and characteristics of coronary artery disease. Furthermore, we aimed to identify the impact of these gene polymorphisms on the evolution of coronary artery disease during the course of statin-based hypolipidaemic treatment in patients stable angina pectoris.
We tested the hypothesis that these eNOS and HO-1 polymorphisms impact the severity and characteristics of coronary artery disease and influence the evolution of coronary atherosclerosis.

2.3. We aimed to determine the utility of the HO-1 promoter (GT)n polymorphism as a predictor of coronary artery disease severity and coronary artery plaque risk profile in patients with stable angina pectoris.

We tested the hypothesis that the HO-1 promoter (GT)n polymorphism, as part of a spectrum on noninvasive tests, will improve the prediction of coronary artery disease severity and coronary artery plaque risk profile.

3. Materials and methods

3.1. Study population

3.1.1. Study population assessed for the influence of the eNOS and HO-1 polymorphisms on the extent and evolution of coronary artery atherosclerosis.

Between November 2005 and April 2009, 70 patients with chronic stable angina were enrolled in the study.

3.1.2 Study population assessed for the utility of the HO-1 promoter polymorphism in the prediction of coronary artery disease severity and plaque risk profile.

Between November 2005 and April 2009, 81 patients with stable angina were included in the study.

3.1.3 Study population assessed for eNOS C-terminal variants

Between September 2008 to September 2009, 150 patients suffering from angiographically documented coronary artery and/or peripheral artery disease were enrolled in the study.

3.2. Molecular genetic methods

3.2.1 Genomic DNA isolation

Patient DNA was isolated from peripheral blood leukocytes using standard techniques.

3.2.2 Molecular genetic analysis of the eNOS and HO-1 genes

ENOS C-terminal variants

The polymerase chain reaction (PCR) was performed using primers designed to amplify exon 25 and 26 of the eNOS gene. Amplification of all samples was performed on the MJ Research DYAD 220 Peltier Thermal Cycler (Conquer Scientific). The amplified exons 25 and 26 were separated by electrophoresis in 1.5% agarose gel. After staining with ethidium bromide, the amplified DNA was purified using the Qiagen Gel Extraction Kit (QIAGEN Ltd). Subsequently, direct sequencing of exon 25 a 26 with forward and reverse primer in all samples was performed on the sequenator Prism ABI (Applied Biosystems). Sequence analysis of exons 25 a 26 with neighboring regions of introns 24, 25 and the 3´-UTR was performed with the Chromas Lite version 2.0 software (Technelysium Pty Ltd).

eNOS exon7 Glu298Asp polymorphism

PCR was performed using primers designed to amplify exon 7 of the eNOS gene. Restriction analysis was performed by incubating three units of *Mbo*I restriction enzyme (Promega, Madison, WI). The restriction products were separated by electrophoresis in 3.8% agarose gel. The analysis of restriction products was performed after the addition of ethidium bromide. The GG (Glu/Glu) variant was classified as being protective, whereas the GT (Glu/Asp) and TT (Asp/Asp) variants were classified as conferring risk.

HO-1 promoter (GT)n polymorphism

HO-1 gene promoter containing a poly (GT)n repeat was amplified by PCR with fluorescently labeled primers. All amplifications were performed in a Dyad thermocycler (BIORAD, Hercules, CA). The PCR product sizes were determined using Li-cor 4200 (LI-COR Biosciences, Lincoln, NE) and ABI PRISM 3130 Genetic Analyzer (Applied Biosystems Carlsbad, CA) DNA sequencers. We used IR700 labeled primers for Li-cor analysis and 6-FAM labeled primers for ABI analysis. The determination of fragment length was accomplished using SagaGT (LI-COR Biosciences) and Peak Scanner™ Software (Applied Biosystems). Selected samples were sequenced in an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems) automated DNA sequencer and then included as size markers in every electrophoresis run. We divided alleles according to the number of GT repeats in two subclasses: < 25 = class S (short) alleles (protective HO-1), and ≥ 25 = class L (long) alleles (risk HO-1). Homozygous class S and heterozygotes were grouped together and compared to homozygous class L carriers.

3.3. Invasive coronary artery assessment

Coronary angiography and intravascular ultrasound

Coronary angiography (CAG) was performed according to standard recommendations. Coronary artery atherosclerotic burden was assessed by means of our own angiographic scoring system (angio score). After CAG, the operator selected a target vessel for intravascular ultrasound (IVUS). Only one coronary artery was investigated in each patient. An IVUS phased-array probe (Eagle Eye 20 MHz 2.9 F monorail), IVUS console, Gold standard software, and automatic pullback (research pullback, model R-l00) were used (Volcano Corp., Rancho Cordova, CA). After administration of 200 μg of intracoronary nitroglycerin, the IVUS probe was introduced into the selected coronary artery beyond a distal fiduciary point (well-defined side branch). Motorized pullback was performed through the

rest of coronary artery. In the follow-up study, patients underwent repeated CAG and IVUS of the same coronary artery after 12 months.

IVUS and virtual histology-IVUS analysis

All measurements were performed in the catheterization laboratory of the General University Hospital of Charles University, Prague, Czech Republic and analyzed by a single operator. Each frame from the virtual histology-IVUS (VH-IVUS) loop was analyzed. The primary IVUS endpoint for plaque magnitude assessment was the percent atheroma volume (PAV). The change of PAV was computed as PAVfollow up – PAVbaseline for each patient. VH-IVUS uses spectral analysis of IVUS radiofrequency data to classify plaque components into four categories: fibrous, fibro-fatty, calcification, and necrotic core (NC). VH-IVUS analyses are reported in relative amounts (percentages of plaque). The virtual histology-derived thin-cap fibroatheroma (VH-TCFA) was defined as a plaque with PAV > 40 % and consisting of more than 10 % of necrotic core in direct contact with the vessel lumen.

3.4 Noninvasive examinations as predictors of coronary artery disease severity and risk profile

Ultrasonographic examination of the carotid arteries

Carotid intima-media thickness (IMT) measurement was performed using B-mode ultrasound with an 8 MHz linear probe on the outlying wall of the common carotid artery (ACC) and the internal carotid artery (ACI- on both sides) in longitudinal sections during end-diastole. We used IMTmass (sum of IMT in ACC and ACI for both sides) and IMTmax (maximum of all IMT measurements in every patient). Experienced ultrasonographers adjudicated carotid plaque presence in the carotid bulb if two of the following three criteria were met: (1) abnormal wall thickness (defined as IMT >1.5 mm); (2) abnormal shape (protrusion into the lumen, loss of alignment with adjacent arterial wall boundary); and (3) abnormal wall texture.

Pro-inflammatory mediators and plasma lipids

The following pro-inflammatory mediators were analyzed by means of the enzyme-linked immunosorbent assay (ELISA): VCAM-1, ICAM-1, TNF-α, CD 40 ligand, high-sensitivity C reactive protein (hsCRP), and IL-6. Furthermore, the complete plasma lipid examination was performed assessing total cholesterol (TC), LDL-C, HDL-C, triglycerides, Apo-A and Apo-B100.

3.5. Statistical analysis

Mean values ± standard deviation or percentages were calculated for all variables. Differences between groups were compared using the $\chi 2$ test. Statistical significance was calculated by Fischer's exact test for categorical variables and by Student's *t*-test for continuous variables. Data were analyzed using JMP 3.2 statistical software (SAS Institute, Cary, NC). A p-value of <0.05 was considered statistically significant. Multivariate statistical analysis was performed using IBM SPSS software version 17.0. Linear regression analysis was used for three dependent variables: angio score, PAV, and NC. Logistic regression was used for the dependent variable VH-TCFA. Optimal groups of predictors were chosen for achieving the best prediction of dependent variables.

4. Results

Paper 1, Folia Biologica 57, 182-190, 2011

The total coronary atherosclerotic burden, as expressed by the angio score, was significantly greater in HO-1 risk variant carriers (HO-1 risk 9.4 ± 4.0 vs. protective 6.9 ± 3.2, p = 0.005), but did not differ with respect to the eNOS genotype (eNOS risk 8.1 ± 3.9 vs. eNOS protective 7.8 ± 3.6, p = 0.69). Similarly, the PAV was significantly higher in carriers of HO-1 promoter risk variant (HO-1 risk 49.3 % ± 6.6 vs. protective 45.0 % ± 5.5, p = 0.004), but did not differ with respect to the eNOS genotype (ENOS risk 46.7 % ± 6.8 vs. protective 46.7 % ± 5.9, p = 0.98). In addition, we found a higher prevalence of the thin cap fibroatheroma (TCFA) in HO-1 risk variant carriers (70.0 % vs. 46.2 %, p =0.047).
A significant interaction between eNOS and HO-1 genotypes and changes in plaque composition was observed, the results are summarized in table 1.

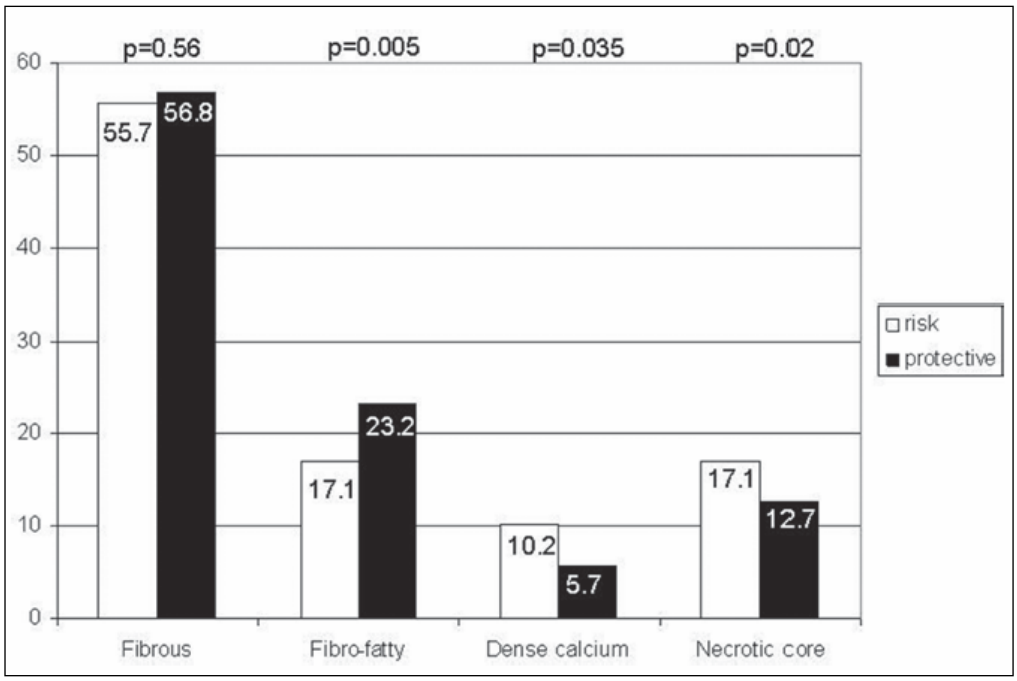
Table 1. Changes of plaque composition according to eNOS and HO-1 genotypes
(Reproduced from Tab. 4 of Král et al., 2011)

Plaque tissue change in %	**Protective ENOS**	**Risk ENOS**	**Protective HO-1**	**Risk HO-1**
Fibrous	-0.3 ± 6.3	-2.4 ± 7.6	- 0.7 ± 8.2	- 2.3 ± 4.8
	p = 0.22		p = 0.34	
Fibro-fatty	-1.0 ± 10.4	-5.1 ± 9.7	- 5.7 ± 10.9	0.9 ± 7.4
	p = 0.09		**p = 0.006**	
Necrotic core	1.3 ± 7.0	3.9 ± 6.7	4.1 ± 7.4	0.4 ± 5.8
	p = 0.1		**p = 0.03**	
Dense calcium	0.5 ± 6.0	3.7 ± 5.3	2,8 ± 6,8	1,3 ± 3,9
	p = 0.02		p = 0.29	

Paper 2, Journal of Invasive Cardiology 25, 32-37, 2013

The risk HO-1 polymorphism was found to influence the extent of coronary atherosclerosis expressed as a higher angio score (9.1 ± 4.2 vs. 6.9 ± 3.1; p = 0.008). Furthermore, risk HO-1 variant carriers showed more prominent plaques, indicated as higher PAV (48.6 ± 6.6% vs. 45.8 ± 5.8%; p = 0.04). In addition, plaques in HO-1 risk variant carriers were characterized by higher proportions of necrotic core (17.1 ± 8.9% vs. 12.7 ± 7.8%; p = 0.02). The proportion of dense calcium and fibro-fatty components of coronary plaques also differed significantly with respect to the HO-1 variant, figure 1. The HO-1 risk polymorphism was found to be a significant predictor of VH-TCFA according to multivariate analysis (data not shown).

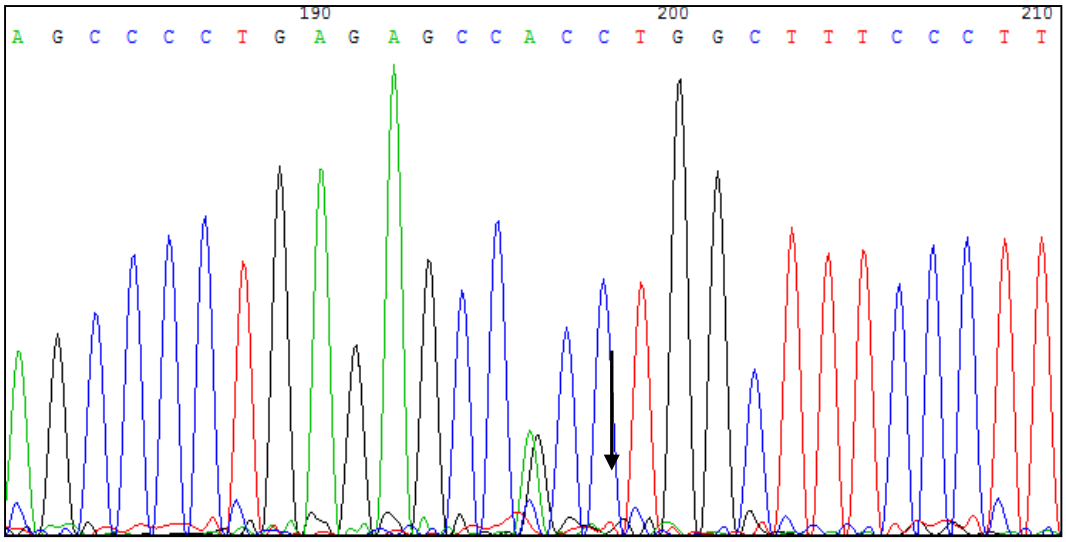
Figure 1. Relationship between HO-1 promoter polymorphism and plaque composition
(Reproduced from Fig. 1 of Kovárník et al. 2013)



Paper 3, article in submission

To test the hypothesis that genetic variants in the C-terminal part of the eNOS gene represent a novel genetic factor contributing to endothelial dysfunction and atherosclerosis development by means of direct sequencing in patients with coronary artery and peripheral artery disease. A novel synonymous G3617C polymorphism in exon 25 was found in one patient. Furthermore, a novel G3911A exon 26 polymorphism leading to a substitution of adenosine (A) for guanosine (G), in the noncoding mRNA (3´-untranslated region-3´-UTR) 6 bp from the stop codon was discovered in one other patient, figure 2.

Figure 2. Novel G3911A variant localized in the noncoding region (3´-UTR) of exon 26 of the eNOS gene



5. Discussion

Endothelial dysfunction is one of the key steps in atherosclerosis initiation and progression, but to date, the genetic factors contributing to endothelial dysfunction and atherosclerosis development are poorly characterized. The eNOS and HO-1 are enzymes crucial for vascular homeostasis and alterations in their regulation and function are involved in atherogenesis. According to current evidence, HO-1 up-regulation during the course of the atherosclerotic disease represents one of the key mechanisms acting to restore NO bioavailability and vascular homeostasis. Numerous genetic association studies have evaluated the relationship of several common eNOS polymorphisms to the risk of endothelial dysfunction and atherosclerosis development, in part, with conflicting results. The eNOS G894T (Glu298Asp) polymorphism has been linked to increased risk of atherosclerosis in multiple studies (Li et al., 2010). Decreased NO production by the variant enzyme under basal conditions and upon agonist stimulation has been attributed to its altered caveolar association and diminished dissociation from Cav-1 in response to shear stress (Joshi et al., 2007). Furthermore, the amino acid substitution at position 298 of the eNOS protein leads to a significant reduction in amino acid distances, thereby possibly affecting protein geometry and thus eNOS protein–protein interactions (Joshi and Bauer, 2008). These alterations seem to

affect eNOS protein function and possibly explain the enhanced disease risk associated with the Asp allele. Similarly, the HO-1 (GT)n promoter polymorphism, shown to profoundly influence HO-1 expression, and hence enzymatic activity, has been linked to increased risk of atherosclerosis in many studies (Araujo et al., 2012).

However, very limited data are available on the impact of these eNOS and HO-1 polymorphisms on the extent and characteristics/risk profile of coronary artery atherosclerotic disease and their influence on the evolution of coronary artery atherosclerosis. Furthermore, little attention has been devoted to the search for variations in exons 25 and 26, encoding the C-terminal part of the eNOS reductase domain, shown to be crucial for proper enzyme function and the 3´-UTR, demonstrated to have a key role in eNOS mRNA stability (Wang and Wang, 2000). We thus sought to evaluate the genetic determinants of endothelial dysfunction and atherosclerosis by assessing the impact of eNOS and HO-1 polymorphisms on coronary artery disease severity and risk profile assessed comprehensively by means of CAG, IVUS and IVUS-VH, and their evolution during hypolipidaemic treatment. In addition, we studied genetic variation in exons 25 and 26 of the eNOS gene, encoding the C-terminus of eNOS and the 3´-UTR.

The key finding of our studies is that patients with the HO-1 risk promoter polymorphism have greater coronary artery atherosclerotic burden and more prominent atherosclerotic plaques. Furthermore, these patients have higher proportion of necrotic tissue in coronary plaques and a higher prevalence of the TCFA type of plaques, i.e. plaque features related to the development of unstable plaques. HO-1 activity is principally regulated at the level of gene transcription, thus, any mechanism affecting the transcriptional activity of the HO-1 gene can profoundly alter HO-1 enzymatic activity. Carriers of the HO-1 long alleles (L) demonstrate decreased HO-1 transcriptional activity upon induction, compared with the short alleles (S) (Taha et al., 2010). According to our findings, the HO-1 (GT)n promoter polymorphism plays an important role in coronary artery atherosclerosis development, progression and possibly in plaque type formation as well. The blunted induction of HO-1 gene transcription in the promoter risk variant carriers resulting in increased oxidative stress presumably accounts for the increased susceptibility of these individuals to the development of more advanced coronary atherosclerosis, such as we have observed in our population. Moreover, our results, as well as other current findings, suggest that adequate HO-1 activity may help to prevent the transformation of a lesion to a high-risk plaque by impeding accretion of the necrotic core and promoting VSMC survival in the plaque fibrous cap (Larsen et al., 2010). HO-1 expression has been shown to correlate with increasing coronary lesion

complexity and stenosis grade and HO-1 expression is up-regulated in vulnerable carotid plaques. Enhanced HO-1 expression in atherosclerotic lesions most likely represents a compensatory atheroprotective and anti-inflammatory response. However, further research is required to clarify why highly up-regulated HO-1 may be overwhelmed and thus fail to halt the progression of atherosclerotic lesions.

Conversely, the eNOS Glu298Asp polymorphism had no impact on the severity of coronary artery atherosclerotic involvement in our cohort. However, we observed an association of this eNOS polymorphism with atherosclerotic plaque composition changes during statin treatment. In patients with the eNOS protective variant (Glu/Glu), we found favorable plaque composition changes characterized by a significantly lower increase in plaque calcified tissue, compared with carriers of the risk variants. Statins lead to enhanced eNOS expression and increase eNOS activity via activation of the Akt kinase pathway and inhibition of Cav-1 expression in endothelial cells (Blum et al., 2009). We speculate that the effects of statins may be more pronounced in the wild-type eNOS enzyme carriers, and therefore, the greater up-regulation of eNOS activity could contribute to the favorable changes in plaque composition observed in these individuals. However, the extent to which our observations were affected by statin treatment remains to be elucidated by future investigations.

Given the crucial role of the C-terminal part of the reductase domain of eNOS for proper enzyme function, we hypothesized that polymorphisms or mutations in the C-terminal part of eNOS could profoundly affect enzyme function and thus play a role in the pathogenesis of endothelial dysfunction and atherosclerosis. However, the sequencing of exons 25 and 26 of the eNOS gene, demonstrated minimal variation in our patient population. No amino acid-altering variants in exons 25 and 26 were detected, nevertheless, a novel variant in the 3′-UTR of exon 26 was found. Posttranscriptional regulation of eNOS mRNA stability is an important component of the immensely complex system of eNOS regulation. A range of mediators involved in atherogenesis have been shown to decrease eNOS mRNA stability via modulation of ribonucleoprotein (RNP) binding to the 3′-UTR of eNOS (Tai et al., 2004). It is plausible that genetic variants in the 3′-UTR could alter the binding affinity of *trans*-acting elements, and thereby influence eNOS mRNA stability. Thus, our finding of a novel variant in the 3′-UTR of the human eNOS gene warrants further investigation of its possible impact on eNOS posttranscriptional regulation.

6. Conclusions

We did not observe an association of the eNOS G894T polymorphism with the extent and risk profile of coronary atherosclerosis. Nevertheless, we observed an association of this eNOS variant with plaque composition changes during hypolipidaemic statin-based therapy. Statins have been shown to increase eNOS expression and activity through multiple mechanisms. We hypothesize that effects of statins on posttranslational eNOS regulation, namely eNOS protein caveolar association and other protein-protein interactions, may be more pronounced in the wild-type eNOS carriers, thus possibly leading to the restoration of adequate eNOS activity contributing to the favorable plaque composition changes we have observed in these individuals.

Our study demonstrated no amino-acid altering variants in the C-terminal part of eNOS in patients with atherosclerosis, a finding that may reflect the pivotal significance of the C-terminus for proper eNOS function, when any changes in sequence would be too deleterious. Given the significance of the 3′-UTR for the regulation of eNOS mRNA stability, our finding of a novel genetic variant in the 3′-UTR of the human eNOS gene warrants further investigation of its possible impact on eNOS posttranscriptional regulation.

According to our findings, the risk HO-1 promoter polymorphism correlates with greater coronary artery atherosclerotic burden and high-risk coronary plaque features, such as more prominent plaques, larger necrotic core proportion and a higher prevalence of the TCFA type of plaque. The observed greater disease burden and higher risk profile of CAD in risk HO-1 variant carriers is attributable to insufficient HO-1 activity resulting in persistent oxidative stress and inflammation in the vascular wall. On the other hand, the protective HO-1 variant did not prevent negative plaque composition changes during statin therapy in our patients, suggesting that adequate HO-1 activity may be overwhelmed in atherosclerosis and thus fail to impede the progression of atherosclerotic lesions.

To the best of our knowledge, no study to date has evaluated the relationship of eNOS and HO-1 genetic variants to coronary atherosclerosis characterized in such a thorough manner and larger-scale studies are warranted to confirm and possibly further extend our findings. In light of our results, HO-1 promoter polymorphism assessment in individuals with suspected coronary artery disease seems desirable, given the unequivocally higher risk of the L/L variant carriers characterized by greater disease burden and high-risk coronary artery plaque features. Thus, we suggest to incorporate the assessment of the HO-1 (GT)n promoter polymorphism into the spectrum of noninvasive tests utilized for coronary artery disease

severity and risk profile prediction. Our findings significantly contribute to the recognition of the genetic background of endothelial dysfunction and atherosclerosis and the elucidation of possible causes of variable vascular responses to hypolipidaemic therapy.

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