Soluble endoglin and hypercholesterolemia aggravates endothelial and vessel wall dysfunction in mouse aorta

Barbora Vitverova^a, Katerina Blazickova^a, Iveta Najmanova^a, Matej Vicen^a, Radek Hyšpler^b, Eva Dolezelova^a, Ivana Nemeckova^a, Jurjen Duintjer Tebbens^c, Carmelo Bernabeu^d, Miguel Pericacho^e, Petr Nachtigal^{a*}

^aDepartment of Biological and Medical Sciences, Faculty of Pharmacy in Hradec Kralove, Charles University, Heyrovskeho 1203, Hradec Kralove, 500 05, Czech Republic

^bCentrum for Research and Development, University Hospital, Hradec Kralove, Czech Republic.

^cDepartment of Biophysics and Physical Chemistry, Faculty of Pharmacy in Hradec Kralove, Charles University, Heyrovskeho 1203, Hradec Kralove, 500 05, Czech Republic

^dCenter for Biological Research, Spanish National Research Council (CSIC), and Biomedical Research Networking Center on Rare Diseases (CIBERER), Madrid, Spain, 28040, Madrid, Spain

^eBiomedical Research Institute of Salamanca (IBSAL), and Renal and Cardiovascular Physiopathology Unit, Department of Physiology and Pharmacology, University of Salamanca, 37007, Salamanca, Spain

*Corresponding author. E-mail address: petr.nachtigal@faf.cuni.cz

Phone/fax numbers: +420495067305/ +420495067170

Number of figures 5.

Abstract

Background and aims

Increased plasma levels of soluble endoglin (sEng) were detected in patients with endothelial dysfunction-related disorders and hypercholesterolemia. In this study, we hypothesized that high levels of sEng accompanied by mild hypercholesterolemia could aggravate endothelial and vessel wall dysfunction and affect endoglin/eNOS signaling in mouse aorta.

Methods

Three-month-old female transgenic mice on CBAxC57BL/6J background with high levels of sEng (*Sol-Eng*⁺ *high HFD*) and their littermates with low levels of sEng (*Sol-Eng*⁺ *low HFD*) were fed with high fat diet for six months. Plasma samples were used for biochemical, ELISA and Luminex analyses of total cholesterol, sEng and inflammatory markers. Functional parameters of aorta were assessed by means of wire myograph 620M. Western blot analyses of membrane endoglin/eNOS signaling and endothelial dysfunction/inflammation markers in aorta were performed.

Results

Functional analysis of aorta showed impaired KCl induced vasoconstriction, endothelial-dependent relaxation after the administration of acetylcholine as well as endothelial-independent relaxation induced by sodium nitroprusside in *Sol-Eng*⁺ *high HFD* group compared to *Sol-Eng*⁺ *low HFD* group. Ach-induced vasodilation after administration of L-NAME was significantly higher in *Sol-Eng*⁺ *high HFD* group compared to *Sol-Eng*⁺ *low HFD* group. The expressions of endoglin, p-eNOS/eNOS, pSmad2/3/Smad2/3 signaling pathway were significantly lower in *Sol-Eng*⁺ *high HFD* group compared to *Sol-Eng*⁺ *low HFD* group.

Conclusions

The results indicate that long-term hypercholesterolemia combined with high levels of sEng leads to the aggravation of endothelial and vessel wall dysfunction in aorta, with possible alteration of membrane endoglin/eNOS signaling suggesting that high levels of soluble endoglin might be considered as a risk factor of cardiovascular diseases.

Keywords: Soluble endoglin; Endothelial dysfunction; eNOS, Vascular reactivity; Mice

1. Introduction

Endoglin (Eng) is a 180-kDa homodimeric transmembrane glycoprotein, predominantly expressed in endothelial cells and playing a key role in cardiovascular development, angiogenesis, vascular remodeling and homeostasis [1, 2]. Eng is a co-receptor of the TGF- β receptor complex that interacts with TGF- β signaling receptors types I and II, and modulates cellular responses to TGF- β [3, 4].

A soluble form of endoglin (sEng) is released from membrane bound Eng by proteolytic cleavage of its extracellular domain during endothelial injury or inflammation and subsequently sEng enters the blood circulation [5, 6]. Increased levels of sEng in plasma have been detected in patients with cardiovascular-related disorders like atherosclerosis [7, 8], hypercholesterolemia [9], hypertension, type II diabetes mellitus [10] and preeclampsia, where sEng levels correlate with disease severity [11]. Because all these pathological conditions are associated with endothelial dysfunction, it was suggested that high levels of sEng could be considered as a biomarker of endothelial dysfunction and cardiovascular-related pathologies [12].

Endothelial dysfunction is considered as the first and key step in the development of atherosclerosis. It is mostly associated with an impairment of endothelial-dependent vasodilation mediated by nitric oxide (NO). The endothelium has a constitutive expression of NO catalyzed by endothelial nitric oxide synthase (eNOS) that is one of the most important factors leading to normal vascular relaxation [13, 14]. Decreased expression of membrane Eng was demonstrated to be associated with lower NO-dependent vasodilation and reduced expression of eNOS [15-17]. In addition, it has been reported that Eng affects eNOS expression in endothelial cells via Smad2/3 transcription factors without involvement of TGF- β [18]. In addition, vascular endothelium controls vasodilation respectively vasoconstriction via myosin light chain kinase signaling in smooth muscles [19].

The phosphorylation of myosin light chain (MLC) is the main regulatory mechanism of smooth muscle contraction [20].

It is of interest to mention, that sEng was demonstrated to have potentially opposite effects on vascular endothelium as compared to membrane endoglin, resulting in inhibition of eNOS-dependent vasodilation [11] or higher expression of cell adhesion molecules [21]. Our previous studies in mice were focused on possible effects of sEng in the atherosclerosis prone aorta under the conditions of chow and high fat diet [22, 23]. sEng alone did not induce any signs of endothelial dysfunction [23] but combination with hypercholesterolemia resulted in development of pro-inflammatory phenotype. However, this pathological condition was not accompanied by the induction of endothelial dysfunction [22].

Thus, in this study, we used the same transgenic mouse model expressing human sEng (*Sol-Eng*⁺) [24] to reveal whether longer exposure to sEng and hypercholesterolemia will result in functional alteration of aorta. We hypothesized that sEng aggravates endothelial and vessel wall dysfunctions in aorta that are primarily triggered by high fat diet.

2. Materials and methods

2.1. Animals and Experimental Design

Transgenic mice overexpressing human sEng (*Sol-Eng*⁺) on the CBAxC57BL/6J background were generated at the Genetically Modified Organisms Generation Unit (University of Salamanca, Spain), as previously described [24]. Six-month-old female mice with low levels of sEng fed a chow rodent diet (*Sol-Eng*⁺ low chow) were used as control mice for biochemical analysis only. All other experiments were performed with three-month-old female mice with high plasma levels of sEng (*Sol-Eng*⁺ high HFD) (mice with sEng levels higher than 100 ng/mL were considered as *Sol-Eng*⁺ high HFD) and their age matched female transgenic littermates with low levels of sEng (*Sol-Eng*⁺ low HFD) fed a high fat rodent diet containing 1.25% of cholesterol and 40% of fat (Research Diets, Inc., USA) for the following 6 months. The animals were kept in controlled ambient conditions in a temperature-controlled room with a 12h-12h light-dark cycle with constant humidity and had access to tap water *ad libitum*. No weight differences were observed among experimental groups.

All experiments were carried out in accordance with the standards established in the directive of the EU (2010/63/EU) and all procedures were approved by the Ethical Committee for the protection of animals against cruelty at Faculty of Pharmacy, Charles University (Permit Number: 21558/2013-2), and the Bioethics Committee of the University of Salamanca (Permit Number: 006-201400038812). Mice were anesthetized with ketamine/xylazine, and all efforts were made to minimize the suffering of the animals.

2.2. ELISA and Luminex assay

Blood samples were obtained from vena cava inferior and plasma levels of human sEng were determined by means of Human Endoglin/CD105 Quantikine ELISA Kit (R&D Systems, MN, USA) according to the manufacturer's instructions. Due to the specificity of

the transgenic mouse model, the methodology of human sEng evaluation in plasma had to be adjusted for our studies (300-fold dilution of murine samples compared to typical 4-fold dilution in standard human studies). Plasma levels of intercellular adhesion molecule 1 (ICAM-1), monocyte chemoattractant protein 1 (MCP-1), P-selectin and tumor necrosis factor α (TNF α) were determined by means of Mouse Premixed Multi-Analyte Magnetic Luminex Kit (R&D Systems, MN, USA), according to the manufacturer's protocol. Concentrations were reported as nanograms per milliliter (ng/mL) for human sEng and picograms per milliliter (pg/mL) for ICAM-1, MCP-1, P-selectin and TNF α .

2.3. Biochemical analysis

Total concentration of plasma cholesterol was measured enzymatically by conventional enzymatic diagnostic kits (Lachema, Brno, Czech Republic) and spectrophotometric analysis (cholesterol at 510 nm, ULTROSPECT III, Pharmacia LKB Biotechnology, Uppsala, Sweden). Concentrations were reported as millimolar (mmol/L).

2.4. Immunohistochemistry

Serial sections of heart were cut until the aortic root containing semilunar valves together with aorta appeared. From this point on, serial cross-sections (7 μ m) were cut on a cryostat and placed on gelatin-coated slides. For fluorescence staining of macrophages/monocytes, slides were incubated with rat anti-mouse monoclonal primary antibody macrophages/monocytes (MOMA-2) (Bio-Rad Laboratories, CA, USA) at a dilution of 1:100 in 1% BSA for 1 hour in room temperature. Goat anti-rat secondary antibody marked with green fluorochrome DyLight488 (Jackson ImmunoResearch, USA) was used at a dilution of 1:100 in 1% BSA for 30 minutes at room temperature to detect macrophages. For nuclear counterstaining, blue-fluorescent DAPI nucleic acid staining was used (Invitrogen, Czech Republic).

Photo documentation and image digitizing were performed by using a digital firewire camera Pixelink PL-A642 (Vitana Corporation, Ottawa, Canada) and a VDS Vosskuehler CD-1300QB monochromatic camera (VDS Vosskühler GmbH, Germany) for fluorescence with image analysis software NIS (Laboratory Imaging, Czech Republic).

2.5. Functional analysis of vascular reactivity

On the day of the experiment, mice were anesthetized i.p. with a mixture of ketamine (100 mg/kg) and xylazine (16 mg/kg) and thoracic aortas were quickly removed, carefully dissected from surrounding tissue and immediately placed in Krebs-Henseleit solution. Connective and adipose tissue in the adventitia was carefully removed, and the descending aorta was cut into 4 rings each approximately 3 mm long. The aortic rings were transferred to organ-bath chambers of the wire myograph (620M, Danish Myo Technology, Denmark) filled with 5 mL of Krebs-Henseleit solution. The rings were mounted between 2 pins, attached to an isometric force transducer with continuous recording of tension (PowerLab, LabChart, ADI Instruments, Australia) and gassed with 95% O₂ and 5% CO₂. The resting tension was stepwise increased to reach final 10 mN and then rings were incubated to equilibrate for 30 min at 37°C. The vascular viability was verified by a contractile response to potassium chloride (KCl, 60 mM). After washing, the aortic rings were pre-contracted with increasing concentrations of prostaglandin F2 alpha (PGF2 α , 0.001-1 μ M) to reach approximately 80% of KCl-induced contraction. After reaching a stable plateau phase, the endothelium-dependent relaxation was induced by a cumulative concentration of acetylcholine (Ach, 0.001-1 μ M). For the endothelium-independent relaxation, a cumulative concentration-dependent response for sodium nitroprusside (SNP, 0.001-1 μ M) was induced. Effect of NO production on vascular reactivity was measured by analyzing the Ach effect in the presence of the nitric oxide synthase inhibitor N (ω)-nitro-L-arginine methyl ester (L-NAME, 300 μ M). Between

each concentration-response curve, the rings were washed three times with Krebs-Henseleit solution and left to stabilize.

2.6. Western blot analysis

Samples of aortas were homogenized in RIPA lysis buffer (Sigma-Aldrich, St. Louis, USA) supplemented with protease (SERVA Electrophoresis, Germany) and phosphatase (Thermo Fisher Scientific Inc., IL, USA) inhibitors, as described previously [23]. Homogenates (20 µg of total protein) were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, transferred onto PVDF membrane (Millipore, NY, USA), and incubated with appropriate antibodies (Supplemental Table 1). Horseradish peroxidase-conjugated secondary antibodies were from Sigma-Aldrich Co. (St. Louis, USA) as described previously [23]. Membranes were developed using enhanced chemiluminescent reagents (Thermo Fisher Scientific Inc., IL, USA) and exposed to X-Ray films (Foma, Czech Republic). Densitometric quantification of immunoreactive bands was performed by image analysis software NIS (Laboratory Imaging, Prague, Czech Republic). Protein specific signals in each lane were normalized to GAPDH signal.

2.7. Statistical analysis

The statistical analysis was performed by GraphPad Prism 7.0 software (GraphPad Software, Inc., CA, USA). All data are presented as mean $\pm$ S.E.M. All multiple comparison data were analyzed using ANOVA with Tukey's multiple comparisons test. Direct group-group comparisons were carried out using non-parametric Mann-Whitney test because D'Agostino & Pearson omnibus normality test failed. P values of 0.05 or less were considered statistically significant.

3. Results

3.1. Plasma concentrations of human sEng, inflammatory markers and total cholesterol in *Sol-Eng*⁺ mice

Mice were fed with high fat diet (*HFD*) for 6 months and subsequently plasma samples were collected. Six-month-old female mice fed a chow rodent diet were used as control group with reference values of the lipid profile parameters. Biochemical analysis of plasma samples demonstrated significant differences in total cholesterol concentration (1.84 ± 0.24 vs. 2.94 ± 0.10 mmol/L) between *Sol-Eng*⁺ *low CHOW* and *Sol-Eng*⁺ *low HFD* mice and between *Sol-Eng*⁺ *high CHOW* and *Sol-Eng*⁺ *high HFD* mice (1.83 ± 0.15 vs. 2.93 ± 0.10 mmol/L) respectively. Significantly higher concentration of total cholesterol was also observed in *Sol-Eng*⁺ *high HFD* mice (3.37 ± 0.23 mmol/L) compared to *Sol-Eng*⁺ *low CHOW* mice (1.84 ± 0.24 mmol/L) and in *Sol-Eng*⁺ *high CHOW* mice (1.83 ± 0.15 mmol/L) compared to *Sol-Eng*⁺ *low HFD* mice (2.93 ± 0.10 mmol/L). On the contrary, no differences were observed between *Sol-Eng*⁺ *low HFD* mice (2.93 ± 0.10 mmol/L) and *Sol-Eng*⁺ *high HFD* mice (2.93 ± 0.10 mmol/L) or between *Sol-Eng*⁺ *low CHOW* mice (1.84 ± 0.24 mmol/L) and *Sol-Eng*⁺ *high CHOW* mice (1.83 ± 0.15 mmol/L) (Fig. 1A). As shown in Fig. 1B, ELISA measurements showed that human sEng plasma concentrations were significantly higher in *Sol-Eng*⁺ *high HFD* mice (2796.00 ± 264.00 ng/mL) compared to *Sol-Eng*⁺ *low HFD* mice (7.40 ± 3.35 ng/mL).

Furthermore, Luminex assay showed no significant differences in levels of inflammatory markers ICAM-1 (4574.00 ± 276.00 vs. 4482.00 ± 664.00 pg/mL) and P-selectin (4434.00 ± 659.00 vs. 3935.00 ± 523.00 pg/mL) between studied groups. On the other hand, MCP-1 levels (88.70 ± 23.40 vs. 45.30 ± 7.67 pg/mL), and TNF α (0.62 ± 0.14 vs. 0.33 ± 0.08 pg/mL) were higher (borderline of statistical significance for MCP [$p=0.08$] and

for TNF- α [$p=0.06$]) in *Sol-Eng⁺ high HFD* when compared to *Sol-Eng⁺ low HFD* female mice, respectively (Fig. 1C, D, E, F).

3.2. Impaired vascular reactivity in aortas from *Sol-Eng⁺ high HFD* mice

KCl, a calcium-sensitizing stimulus leading to increased cytosolic free Ca^{2+} concentration, was used in concentration 60 mM to assess the contractile maximal response. As shown in Fig. 2A, the maximal contraction achieved in response to KCl was significantly lower in *Sol-Eng⁺ high HFD* compared to *Sol-Eng⁺ low HFD* mice (2.83 ± 0.20 vs. 3.41 ± 0.20 , respectively). PGF2 α , a receptor-dependent vasoconstrictor, was used for pre-contraction of aortic rings prior to subsequent measurements. The maximal induced contraction after administration of PGF2 α (1 μM) was not significantly different between *Sol-Eng⁺ high HFD* and *Sol-Eng⁺ low HFD* mice ($64.70 \pm 9.26\%$ vs. $85.99 \pm 14.18\%$, respectively) (Fig. 2B).

For endothelium-dependent relaxation, the vasodilator response in PGF2 α pre-contracted aortic rings were induced by acetylcholine, a substance that mediates releasing of NO by binding to muscarinic receptors in endothelial cells. Significantly impaired relaxation was measured at 1 μM acetylcholine ($8.71 \pm 2.68\%$ vs. $21.00 \pm 5.11\%$, respectively) in *Sol-Eng⁺ high HFD* compared to *Sol-Eng⁺ low HFD* mice (Fig. 2C). Endothelium-independent relaxation induced by SNP, a donor of NO with capability to activate soluble guanylate cyclase in vascular smooth muscle, in PGF2 α pre-contracted aortic rings was significantly impaired at 1 μM SNP ($12.90 \pm 1.96\%$ vs. $23.50 \pm 1.42\%$, respectively) in *Sol-Eng⁺ high HFD* compared to *Sol-Eng⁺ low HFD* mice (Fig. 2D) as well. To evaluate whether vasodilation induced by acetylcholine was entirely eNOS-dependent, L-NAME as a direct inhibitor of eNOS was used. The effect of L-NAME on Ach-induced relaxation in PGF2 α pre-constricted vessels reached significantly higher values of vasodilation at 1 μM Ach ($40.90 \pm 15.10\%$ vs. $3.14 \pm 1.30\%$, respectively) in *Sol-Eng⁺ high*

HFD compared to *Sol-Eng⁺ low HFD* mice (Fig. 2E), suggesting that Ach-induced vasodilation response was accompanied by additional compensatory NO releasing mechanisms. It is of interest to mention that endothelium-dependent relaxation and endothelium-independent relaxation induced by SNP were significantly lower compared to our previous study in mice fed by high fat diet for three months [22] suggesting alteration of endothelial function by high fat diet in both studied groups in the paper.

3.3. *Sol-Eng⁺ high HFD* mice do not develop pro-inflammatory phenotype in aorta

Western blot analysis of inflammatory markers related to endothelial dysfunction was performed. No significant differences between *Sol-Eng⁺ high HFD* and *Sol-Eng⁺ low HFD* mice in the expression of VCAM-1 (Fig. 3A), ICAM-1 (Fig. 3B) and P-selectin (Fig. 3C) were observed.

The activation of nuclear factor kappa B (NF- κ B) is reported as a ratio of phosphorylated (active) form expression to the non-phosphorylated form expression. The activation of NF- κ B was not significantly different between *Sol-Eng⁺ high HFD* and *Sol-Eng⁺ low HFD* mice as well (Fig. 3D).

3.4. Endoglin/eNOS signaling is altered in *Sol-Eng⁺ high HFD* mice

Regarding the fact that membrane endoglin signaling via Smad2/3 transcription factor was previously shown to be important in endothelial function, we analyzed the expression of membrane Eng, eNOS and its phosphorylated form p-eNOS Ser1177, as well as Smad2/3 and its phosphorylated form.

The expressions of eNOS and p-eNOS (Ser1177), Smad2/3 and pSmad2/3, are reported as a ratio of the phosphorylated form expression to its non-phosphorylated form expression. Significantly reduced expressions of membrane Eng (to 73%; Fig. 4A), p-eNOS

Ser1177/e-NOS (to 55%; Fig. 4B) and pSmad2/3/Smad2/3 (to 63%; Fig. 4C) were observed in aortas of *Sol-Eng*⁺ *high HFD* mice compared to *Sol-Eng*⁺ *low HFD* mice.

3.5. *Sol-Eng*⁺ *high HFD* mice have impaired vasoconstriction in aorta but no prostacyclin synthase/cyclooxygenase type 2 compensation of altered eNOS-dependent vasodilatation.

Myosin light chain (MLC) is a major regulatory component in vascular smooth muscle contraction. To determine the phosphorylation (activation) of MLC, expression of phosphorylated MLC on residues threonine 18 and serine 19 (pMLC Thr18/Ser19) was assessed, as shown in Fig. 5A. Significantly lower expression of pMLC Thr18/19 (by 76%) was demonstrated in aortas of *Sol-Eng*⁺ *high HFD* mice compared to *Sol-Eng*⁺ *low HFD* mice.

Expression of prostaglandin I₂ synthase (PTGIS) also known as prostacyclin synthase (Fig. 5B) and cyclooxygenase type 2 (COX-2) (Fig. 5C) was measured to reveal potential compensation mechanisms leading to release of NO under pathological conditions. However, no statistical significant changes were observed in the expressions of either PTGIS or COX-2.

3.6. Immunohistochemical analysis of macrophages in aortic sections

Sixty aortic sections in thirty slides were evaluated per each experimental group. The aortic sections did not develop alteration in histological structure with respect to possible fat accumulation (fatty streaks) or other visible changes in the vessel wall (data not shown). In addition, no infiltration of macrophages in the vessel wall was observed either in *Sol-Eng*⁺ *high HFD* mice or *Sol-Eng*⁺ *low HFD* mice. *ApoE*/*LDLR* double knockout mice aortic section was used as a positive control for macrophage staining in aorta (Supplemental Fig. 1).

4. Discussion

Hypercholesterolemia, atherosclerosis, type II diabetes mellitus and hypertension are pathological disorders that were demonstrated to be related to increased levels of sEng, suggesting that sEng could be a potential biomarker of endothelial alteration, which is typical for above mentioned diseases [12]. However, the question is whether sEng might also modulate the function of membrane-bound endothelial endoglin and endothelial function in these pathological conditions, which are usually accompanied by the hypercholesterolemia. Of note, hypercholesterolemia is associated with increased levels of oxysterols, which via LXR nuclear receptors can upregulate the expression of both membrane-bound and soluble endoglin [24, 25]. Here we demonstrate for the first time that long exposure to high sEng levels and mild hypercholesterolemia (induced by high fat diet) result in endothelial and vessel wall dysfunction in aorta and altered membrane endoglin/eNOS signaling.

sEng is chipped from the extracellular domain of the membrane-bound endoglin, which is expressed by endothelial cells and smooth muscle cells in blood vessels [6]. In addition, several papers demonstrated that sEng can directly affect the function of blood vessels. Thus, it has been shown that administration of recombinant sEng led to impaired binding of TGF- β 1 to its receptor and subsequently to an inhibition of eNOS-dependent vasodilation in rat renal microvessels and mesenteric vessels, suggesting possible hypertensive effects of sEng [11]. It is of interest to point out that mice treated with adenovirus expressing sEng had induction of endothelial dysfunction profile in endothelium of mesenteric venules, which was characterized by higher expression of cell adhesion molecules and impaired vasodilation [21]. As these studies were not actually related to atherosclerosis prone blood vessels, we performed the first study that was targeted to elucidate potential effect of high levels of sEng on aorta [23]. By using transgenic mouse model expressing human sEng (*Sol-Eng*⁺) that shows many symptoms of preeclampsia, such

as hypertension, small pup size, proteinuria and renal damage [24], we failed to detect any harmful effect of sEng on aortic function or expression of endothelial dysfunction related markers [23]. These results are in line with a recent paper showing that sEng does not affect TGF- β signaling alone, but rather together with other TGF- β receptors [26]. To further pursue this line of research, we decided to combine high fat diet with high levels of sEng in order to mimic situation in patients with atherosclerosis, type II diabetes mellitus and hypertension where hypercholesterolemia is very common. The results of Jezkova et al. paper showed that combination with high fat diet induces a pro-inflammatory phenotype in aorta with increased expression of NF- κ B and cell adhesion molecules. Surprisingly, we also found a preservation of aortic function in mice having high sEng plasma levels [22]. Despite these controversial results, we demonstrated that hypercholesterolemia in combination with high plasma levels of sEng deserves further study.

Thus, we hypothesized that long-term exposure to high fat diet in combination with high plasma levels of sEng will result in alteration of aortic function. Indeed, six months feeding with high fat diet resulted in a) impaired endothelium-dependent relaxation induced by acetylcholine (that mediates releasing of NO by binding to muscarinic receptors in endothelial cells), b) impaired endothelium-independent relaxation induced by SNP (a donor of NO with capability to activate soluble guanylate cyclase in vascular smooth muscle cells), c) activated compensatory reaction (measured by L-NAME as direct inhibitor of eNOS), d) reduced contraction induced by KCl and e) reduced phosphorylation of myosin light chain in *Sol-Eng⁺ high HFD* mice. All these functional changes suggest the alteration of both endothelial and vessel wall (smooth muscle cells) function. It is of interest to point out that *Sol-Eng⁺ low HFD* mice showed lower levels of all measured parameters when compared to our previous experiment with mice on chow diet [23] or in mice fed three months with high fat diet [22], suggesting that administration of high fat diet for six months impairs the function

of endothelium and vessel wall itself. Thus, we propose that high sEng plasma levels in combination with hypercholesterolemia aggravate already present endothelial and vessel wall dysfunction in aorta.

Indeed, we must emphasize that cholesterol levels were not significantly different between *Sol-Eng*⁺ *high HFD* and *Sol-Eng*⁺ *low HFD* mice but significant hypercholesterolemia was present in mice fed high fat diet when compared to mice on chow diet. Moreover, histological and immunohistochemical analysis showed no signs of ongoing atherogenesis on morphological level, therefore all effects and results of this study are related to atherosclerosis-intact aorta. So, the only difference between these groups was in the levels of human sEng. In general, there is a high level of homology between mouse and human sEng (99% sequence overlap, 69% identity) [27, 28] and mouse models used by our group showed several times that human sEng can affect mouse endoglin signaling [22, 24]. As mentioned above, there are controversial findings on whether sEng interferes with TGF- β signaling [26, 29]. However, it was demonstrated that sEng antagonizes the effects of membrane endoglin that is highly expressed by endothelial and smooth muscle cells [30]. In addition, we found that protein levels of mouse membrane endoglin are decreased in *Sol-Eng*⁺ *high HFD* mice compared to *Sol-Eng*⁺ *low HFD* mice (Fig. 4A), suggesting a complex and regulatory interplay between the two forms of endoglin. Interestingly, membrane endoglin was shown to be related to a proper function of eNOS and NO-dependent vasodilatation [15, 18], whereas its reduced expression resulted in an impaired endothelium-dependent vasodilatation [15]. In addition, membrane endoglin increases eNOS expression via Smad2/3 transcription factor directly without cooperation with TGF- β 1 [18]. Indeed, Western blot analysis revealed reduced expression of membrane endoglin, phosphorylated Smad2/3 and phosphorylated eNOS Ser1177 in *Sol-Eng*⁺ *high HFD* mice in this study. Thus, we might propose that sEng

aggravates endothelial and vessel wall dysfunction in aorta via interference with membrane endoglin/Smad2/3/eNOS dependent pathway.

Functional analysis of aorta showed activation of some compensatory reaction in aorta of *Sol-Eng⁺ high HFD* mice (measured by L-NAME as direct inhibitor of eNOS). Indeed, there are several ways that could explain this compensatory reaction including activation of COX-2, PGs, or EDHF [31-33]. However, COX-2 and PTGIS protein expression in aorta did not differ between studied groups in this study. On the other hand, we cannot rule out the involvement of COX-2 and PTGIS activity in the compensation based on the protein expression analysis, therefore no clear conclusion can be drawn from these results. In addition, we must point out that we have not deepened more into this compensatory mechanism as this was not the primary goal of this study. In our view, this is a complex issue and further and independent studies are necessary to address this point, which is out of the scope of the manuscript.

We also focused on potential molecular mechanism related to altered vasoconstriction response in *Sol-Eng⁺ high HFD* mice. Vasodilation and vasoconstriction is regulated via myosin light chain kinase signaling in smooth muscle cells [19]. KCl acts as a calcium sensitizer and membrane depolarization occurs after its administration. KCl activates voltage-dependent calcium channel and this leads to an increased intracellular calcium concentration and subsequently to activation of myosin light chain kinase (MLCK) [34]. MLCK is a Ca^{2+} /calmodulin-dependent protein kinase responsible for myosin phosphorylation and the phosphorylation of myosin light chain (MLC) is the main regulatory mechanism of smooth muscle contraction [20]. So, there is a direct relation between activity of MLCK, degree of MLC phosphorylation and therefore ability of smooth muscle contraction [35]. In agreement with this, we showed significantly reduced expression of pMLC Thr18/19 and reduced vasoconstriction in aortas of *Sol-Eng⁺ high HFD* mice compared to *Sol-Eng⁺ low HFD* mice.

Thus, we might propose that 6 months HFD feeding and exposure to high levels of soluble endoglin leads to impaired calcium sensitivity/release and subsequently to decreased capability of smooth muscle contraction in *Sol-Eng⁺ high HFD*.

A key aspect of endothelial dysfunction development is the activation of vascular inflammation with increased expression of cell adhesion molecules [36]. Surprisingly, we did not detect any significant differences in the expression of VCAM-1, ICAM-1, P-selectin and pNF- κ B/NF- κ B in both aorta and blood between both studied groups as we showed in our previous paper [22]. One possible explanation why we do not see the differences in VCAM-1, ICAM-1, P-selectin and pNF- κ B/NF- κ B expressions might be in the fact that mice are under long term high fat diet (6 months) in this study and the expression levels of these proteins might be high enough in both groups (especially considering the fact that both groups of mice have aggravated function of endothelium when compared with Jezkova et al. paper). Thus, different age of mice (9 months), different exposure to high fat diet (6 months) and soluble endoglin (9 months) in this study when compared to Jezkova et al paper (age of mice 6 months, high fat diet 3 months and soluble endoglin 6 months) could be responsible for the different results that we obtained [22]. On the other hand, we measured other pro-inflammatory biomarkers in plasma. We demonstrated increased level of MCP-1 and TNF- α (borderline of statistical significance for MCP-1 [p=0.08] and for TNF- α [p=0.06]) in *Sol-Eng⁺ high HFD* mice suggesting at least partial induced pro-inflammatory reaction in these mice.

It is of interest to mention potential limitation of the study. At this point we cannot answer important question, whether high soluble endoglin levels affect atherogenesis/atherosclerosis in aorta. The group of Lopez-Novoa tried to generate mice with high soluble endoglin on ApoE background, however they failed to generate viable mice who would have high levels of soluble endoglin (unpublished results). So, *in vivo* experiments

investigating atherogenesis in the mice with high levels of soluble endoglin do not appear yet to be feasible. Moreover, we would like to also point out that, we measured blood pressure in Nemeckova et al paper [37]. The measurements of arterial pressure by tail-cuff method showed that systolic pressure in male and female Sol-Eng+ mice is higher than that of control mice. Higher blood pressure in mice with high soluble endoglin is the key feature of this mouse model that we have established before.

In conclusion, we demonstrated that long-term hypercholesterolemia (induced by high fat diet) combined with high plasma levels of sEng results in the aggravation of endothelial and vessel wall dysfunction in aorta with possible alteration of membrane endoglin/eNOS signaling. Thus, it is necessary to consider sEng as not only a biomarker of cardiovascular diseases but also as a potential co-inducer of vascular alterations especially in combination with other risk factors of cardiovascular diseases including hypercholesterolemia.

Conflict of interest

The authors declared they do not have anything to disclose regarding conflict of interest with respect to this manuscript.

Financial support

The work was supported by grants from Czech Science foundation (GACR number GA15-24015S), *Czech Health Research Council* (AZV CR number 17-31754A), the Grant Agency of Charles University in Prague (number 1284214/C, 1158413C and grant SVV/2016/260293), *Ministerio de Economía y Competitividad* of Spain (SAF2013-43421-R to CB *Centro de Investigación Biomédica en Red de Enfermedades Raras* (CIBERER; to CB), and *Instituto de Salud Carlos III* (PI16/00460 and Retic RD16/0009/0025, REDINREN-FEDER). CIBERER and REDINREN are initiatives of the *Instituto de Salud Carlos III* (ISCIII) of Spain supported by FEDER funds from the European Commission.

Author contributions

Barbora Vitverova - designed and performed experiments, wrote the manuscript

Katerina Blazickova - performed experiments, revised the text of the manuscript

Iveta Najmanova - performed myograph experiments, revised the text of the manuscript

Matej Vicen - participated on statistical analysis, revised the text of the manuscript

Radek Hyšpler - performed biochemical analysis of total cholesterol

Eva Dolezelova - contributed to data analysis, revised the text of the manuscript

Ivana Nemeckova – provided cholesterol levels data, revised the text of the manuscript

Jurjen Duintjer Tebbens - performed statistical analysis

Carmelo Bernabeu - revised the text of the manuscript

Miguel Pericacho - revised the text of the manuscript

Petr Nachtigal - designed experiments, wrote and revised the manuscript

Acknowledgements

We thank Prof. Lopez-Novoa from University of Salamanca in Spain kindly providing of unique transgenic mouse model.

References

- [1] Bot PT, Hoefer IE, Sluijter JP *et al.* Increased expression of the transforming growth factor-beta signaling pathway, endoglin, and early growth response-1 in stable plaques. *Stroke* 2009; 40:439-447.
- [2] Lopez-Novoa JM, Bernabeu C. The physiological role of endoglin in the cardiovascular system. *Am J Physiol Heart Circ Physiol* 2010; 299:H959-974.
- [3] Blanco FJ, Santibanez JF, Guerrero-Esteo M *et al.* Interaction and functional interplay between endoglin and ALK-1, two components of the endothelial transforming growth factor-beta receptor complex. *J Cell Physiol* 2005; 204:574-584.
- [4] Guerrero-Esteo M, Sanchez-Elsner T, Letamendia A, Bernabeu C. Extracellular and cytoplasmic domains of endoglin interact with the transforming growth factor-beta receptors I and II. *J Biol Chem* 2002; 277:29197-29209.
- [5] Gallardo-Vara E, Blanco FJ, Roque M *et al.* Transcription factor KLF6 upregulates expression of metalloprotease MMP14 and subsequent release of soluble endoglin during vascular injury. *Angiogenesis* 2016; 19:155-171.
- [6] Hawinkels LJ, Kuiper P, Wiercinska E *et al.* Matrix metalloproteinase-14 (MT1-MMP)-mediated endoglin shedding inhibits tumor angiogenesis. *Cancer Res* 2010; 70:4141-4150.
- [7] Blann AD, Wang JM, Wilson PB, Kumar S. Serum levels of the TGF-beta receptor are increased in atherosclerosis. *Atherosclerosis* 1996; 120:221-226.
- [8] Strasky Z, Vecerova L, Rathouska J *et al.* Cholesterol effects on endoglin and its downstream pathways in ApoE/LDLR double knockout mice. *Circ J* 2011; 75:1747-1755.
- [9] Blaha M, Cermanova M, Blaha V *et al.* Elevated serum soluble endoglin (sCD105) decreased during extracorporeal elimination therapy for familial hypercholesterolemia. *Atherosclerosis* 2008; 197:264-270.

- [10] Blazquez-Medela AM, Garcia-Ortiz L, Gomez-Marcos MA *et al.* Increased plasma soluble endoglin levels as an indicator of cardiovascular alterations in hypertensive and diabetic patients. BMC Med 2010; 8:86.
- [11] Venkatesha S, Toporsian M, Lam C *et al.* Soluble endoglin contributes to the pathogenesis of preeclampsia. Nat Med 2006; 12:642-649.
- [12] Rathouska J, Jezkova K, Nemeckova I, Nachtigal P. Soluble endoglin, hypercholesterolemia and endothelial dysfunction. In: Atherosclerosis. 2015. pp. 383-388.
- [13] Davignon J, Ganz P. Role of endothelial dysfunction in atherosclerosis. Circulation 2004; 109:III27-32.
- [14] Chatterjee A, Black SM, Catravas JD. Endothelial nitric oxide (NO) and its pathophysiologic regulation. Vascul Pharmacol 2008; 49:134-140.
- [15] Jerkic M, Rivas-Elena JV, Prieto M *et al.* Endoglin regulates nitric oxide-dependent vasodilatation. FASEB J 2004; 18:609-611.
- [16] Jerkic M, Rodriguez-Barbero A, Prieto M *et al.* Reduced angiogenic responses in adult Endoglin heterozygous mice. Cardiovasc Res 2006; 69:845-854.
- [17] Toporsian M, Gros R, Kabir MG *et al.* A role for endoglin in coupling eNOS activity and regulating vascular tone revealed in hereditary hemorrhagic telangiectasia. Circ Res 2005; 96:684-692.
- [18] Santibanez JF, Letamendia A, Perez-Barriocanal F *et al.* Endoglin increases eNOS expression by modulating Smad2 protein levels and Smad2-dependent TGF-beta signaling. J Cell Physiol 2007; 210:456-468.
- [19] Gao N, Huang J, He W *et al.* Signaling through myosin light chain kinase in smooth muscles. J Biol Chem 2013; 288:7596-7605.

- [20] Shirazi A, Iizuka K, Fadden P *et al.* Purification and characterization of the mammalian myosin light chain phosphatase holoenzyme. The differential effects of the holoenzyme and its subunits on smooth muscle. *J Biol Chem* 1994; 269:31598-31606.
- [21] Walshe TE, Dole VS, Maharaj AS *et al.* Inhibition of VEGF or TGF- β signaling activates endothelium and increases leukocyte rolling. *Arterioscler Thromb Vasc Biol* 2009; 29:1185-1192.
- [22] Jezkova K, Rathouska J, Nemeckova I *et al.* High Levels of Soluble Endoglin Induce a Proinflammatory and Oxidative-Stress Phenotype Associated with Preserved NO-Dependent Vasodilatation in Aortas from Mice Fed a High-Fat Diet. *J Vasc Res* 2016; 53:149-162.
- [23] Nemeckova I, Serwaczak, A., Oujo, B. *et al.* High soluble endoglin levels do not induce endothelial dysfunction in mouse aorta. In: *PLoS One*. 2015. p. e0119665.
- [24] Valbuena-Diez AC, Blanco FJ, Oujo B *et al.* Oxysterol-induced soluble endoglin release and its involvement in hypertension. *Circulation* 2012; 126:2612-2624.
- [25] Henry-Berger J, Mouzat K, Baron S *et al.* Endoglin (CD105) expression is regulated by the liver X receptor alpha (NR1H3) in human trophoblast cell line JAR. *Biol Reprod* 2008; 78:968-975.
- [26] Wang Q. Molecular genetics of coronary artery disease. *Curr Opin Cardiol* 2005; 20:182-188.
- [27] Gougos A, Letarte M. Primary structure of endoglin, an RGD-containing glycoprotein of human endothelial cells. *J Biol Chem* 1990; 265:8361-8364.
- [28] St-Jacques S, Cymerman U, Pece N, Letarte M. Molecular characterization and in situ localization of murine endoglin reveal that it is a transforming growth factor-beta binding protein of endothelial and stromal cells. *Endocrinology* 1994; 134:2645-2657.
- [29] Gregory AL, Xu G, Sotov V, Letarte M. Review: the enigmatic role of endoglin in the placenta. *Placenta* 2014; 35 Suppl:S93-99.

- [30] Rossi E, Sanz-Rodriguez F, Eleno N *et al.* Endothelial endoglin is involved in inflammation: role in leukocyte adhesion and transmigration. *Blood* 2013; 121:403-415.
- [31] Huang A, Sun D, Carroll MA *et al.* EDHF mediates flow-induced dilation in skeletal muscle arterioles of female eNOS-KO mice. *Am J Physiol Heart Circ Physiol* 2001; 280:H2462-2469.
- [32] Huang A, Sun D, Smith CJ *et al.* In eNOS knockout mice skeletal muscle arteriolar dilation to acetylcholine is mediated by EDHF. *Am J Physiol Heart Circ Physiol* 2000; 278:H762-768.
- [33] Silva BR, Pernomian L, De Paula TD *et al.* Endothelial nitric oxide synthase and cyclooxygenase are activated by hydrogen peroxide in renal hypertensive rat aorta. *Eur J Pharmacol* 2017; 814:87-94.
- [34] Ratz PH, Berg KM, Urban NH, Miner AS. Regulation of smooth muscle calcium sensitivity: KCl as a calcium-sensitizing stimulus. *Am J Physiol Cell Physiol* 2005; 288:C769-783.
- [35] Niirō N, Ikebe M. Zipper-interacting protein kinase induces Ca(2+)-free smooth muscle contraction via myosin light chain phosphorylation. *J Biol Chem* 2001; 276:29567-29574.
- [36] Ling S, Nheu L, Komesaroff PA. Cell adhesion molecules as pharmaceutical target in atherosclerosis. *Mini reviews in medicinal chemistry* 2012; 12:175-183.
- [37] Nemeckova I, Serwaczak A, Oujo B *et al.* High soluble endoglin levels do not induce endothelial dysfunction in mouse aorta. *PLoS One* 2015; 10:e0119665.

FIGURE LEGENDS

Fig. 1: Total cholesterol, soluble endoglin and inflammatory markers in plasma. Plasma concentration of total cholesterol (A) in *Sol-Eng*⁺ *high CHOW* and *Sol-Eng*⁺ *low CHOW* mice fed chow rodent diet and in *Sol-Eng*⁺ *high HFD* and *Sol-Eng*⁺ *low HFD* mice fed high fat diet for 6 months. Human sEng (B), ICAM-1 (C), MCP-1 (D), P-selectin (E) and TNF α (F) in *Sol-Eng*⁺ *high HFD* and *Sol-Eng*⁺ *low HFD* mice fed high fat diet for 6 months. Data are shown as mean $\pm$ S.E.M. Results in Fig. 1A are analyzed by ANOVA, followed by Tukey's multiple comparisons test** $p \leq 0.01$, *** $p \leq 0.001$. Following data are analyzed by Mann-Whitney test, *** $p \leq 0.001$. n = 8 mice per group in all graphs in Figure 1.

Fig. 2: Functional analysis of vascular reactivity by wire myograph in *Sol-Eng*⁺ *high HFD* and *Sol-Eng*⁺ *low HFD* mice. Maximal contraction to KCl (60 mM) (A). Maximal contraction to PGF2 α (1 μ M) (B). Acetylcholine-induced relaxation (1 μ M) in PGF2 α pre-constricted aortic rings (C). SNP-induced relaxation (1 μ M) in PGF2 α pre-constricted aortic rings (D). Inhibitory effect of L-NAME (at 1 μ M Ach) on eNOS-dependent Ach-induced relaxation in PGF2 α pre-constricted aortic rings (E). Data are shown as mean $\pm$ S.E.M. We used Mann-Whitney U-tests (one-tailed, lower KCl, Ach and SNP means, resp. higher L-NAME means in *Sol-Eng*⁺ *low HFD* than in *Sol-Eng*⁺ *high HFD* are considered physically highly improbable), * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$. n = 8 mice per group.

Fig. 3: The expression of inflammatory markers in aorta of *Sol-Eng*⁺ *high HFD* and *Sol-Eng*⁺ *low HFD* mice. The expression of VCAM-1 (A), ICAM-1 (B), P-selectin (C) and pNF- κ B/NF- κ B (D) in total protein extracts from mice aortas. Top: densitometric analysis (control = 100%). Densitometric quantification of immunoreactive bands was recalculated to

GAPDH signal. Bottom: representative immunoblots. Data are shown as mean $\pm$ S.E.M., Mann-Whitney test. n = 5 mice per group.

Fig. 4: The expression of membrane endoglin, eNOS and Smads in aortas from *Sol-Eng*⁺ *high HFD* and *Sol-Eng*⁺ *low HFD* mice. The expression of membrane Eng (A), p-eNOS (Ser1177)/eNOS (B) and pSmad2/3/Smad2/3 (C) in total protein extracts from mouse aortas. Top: densitometric analysis (control = 100%). Densitometric quantification of immunoreactive bands was recalculated to GAPDH signal. Bottom: representative immunoblots. Data are shown as mean $\pm$ S.E.M., Mann-Whitney test, * $p \leq 0.05$, ** $p \leq 0.01$. n = 5 mice per group.

Fig. 5: The expression of markers related to vascular smooth muscle function in aorta of *Sol-Eng*⁺ *high HFD* and *Sol-Eng*⁺ *low HFD* mice. The expression of pMLC Thr18/Ser19 (A), PTGIS (B) and COX-2 (C) in total protein extracts from mouse aortas. Top: densitometric analysis (control = 100%). Densitometric quantification of immunoreactive bands was recalculated to GAPDH signal. Bottom: representative immunoblots. Data are shown as mean $\pm$ S.E.M., Mann-Whitney test, * $p \leq 0.05$. n = 5 mice per group.

Supplemental Fig. S1: Fluorescence staining of macrophages in aorta of *Sol-Eng*⁺ *high HFD* and *Sol-Eng*⁺ *low HFD* mice. No infiltration of macrophages was detected in *Sol-Eng*⁺ *high HFD* mice (A) and *Sol-Eng*⁺ *low HFD* mice (B). Positive staining for macrophages (arrows) in *ApoE*/*LDLR* double knockout mouse aorta (C). Bar, 100 μ m.

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

Supplemental Figure S1