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Endoglin is not expressed with cell adhesion molecules in aorta during atherogenesis in apoE-deficient mice

Jana Rathouska*, Katerina Jezkova*, Ivana Nemeckova, Lenka Zemankova, Michala Varejckova and Petr Nachtigal

Department of Biological and Medical Sciences, Faculty of Pharmacy in Hradec Kralove, Charles University in Prague, Prague, Czech Republic

*These authors contributed equally to this work

Summary. Endoglin (TGF-β receptor III), has been demonstrated to affect vascular endothelium and atherosclerosis. Moreover, it was also demonstrated that endoglin is involved in inflammation and plays a role in leukocyte adhesion and transmigration *in vitro* and *in vivo* but not in atherosclerosis related vessels. In this study, we wanted to evaluate endoglin expression in two different parts of the aorta (heart aortic sinus and ascending aorta) and assess its potential simultaneous expression with cell adhesion molecules in non-atherosclerotic and atherosclerotic aortas of apoE-deficient mice.

Ten-week–old female apolipoprotein E-deficient mice on a C57BL/6J background (n=24) were randomly subdivided into three groups and were fed either chow diet (for another two months) or Western type diet (for another two or four months). Immunohistochemical staining of endoglin, VCAM-1 and P-selectin in aortic sinus and ascending aorta was performed.

Endoglin expression was detected only in endothelial cells and varied during atherogenic process in aorta but not in aortic sinus. Moreover, its expression seemed to be weaker in aorta when compared to aortic sinus and the positivity was detected only in endothelium covering atherosclerotic lesions but not in non-atherosclerotic endothelium regardless of the plaque size. Endoglin was not expressed with P-selectin and VCAM-1 in aortic endothelium in any studied group.

This study shows that endothelial expression of endoglin is related to the atherogenic process predominantly in aorta outside the heart. Moreover, endoglin is not localized with cell adhesion molecules involved in atherosclerosis, suggesting it might not participate in leukocyte accumulation in aorta of apoE-deficient mice during atherogenesis.

Key words: Endoglin, P-selectin, VCAM-1, Atherogenesis, apoE-deficient mice

Offprint requests to: Nachtigal Petr, Department of Biological and Medical Sciences, Faculty of Pharmacy in Hradec Kralove, Charles University in Prague, Heyrovskeho 1203, Hradec Kralove 500 05, Czesch Republic. e-mail: Petr.Nachtigal@faf.cuni.cz

Introduction

Endoglin, an accessory receptor of the TGF-β signaling pathway is a homodimeric transmembrane glycoprotein that has been demonstrated to play a role in vascular physiology and pathology (Lopez-Novoa and Bernabeu, 2010). Changes of endoglin expression and function were detected in hereditary hemorrhagic telangiectasia, preeclampsia, hypertension, cancer and atherosclerosis (Levine et al., 2006; ten Dijke et al., 2008; Perez-Gomez et al., 2010; Nachtigal et al., 2012).

Endoglin expression was detected in atherosclerosis prone vessels in both humans and experimental animals. Endothelial cells (Nachtigal et al., 2009a), smooth muscle cells (Conley et al., 2000) and even macrophages (Piao and Tokunaga, 2006) are able to express endoglin in different stages of the atherosclerotic process. In addition, endoglin is able to affect the expression and activity of endothelial nitric oxide synthase (eNOS), including vasodilatation/vasoconstriction of arteries (Jerkic et al., 2004), production of collagen in vessels by

smooth muscle cells (Bot et al., 2009) and formation of new blood vessels in atherosclerotic plaques (Luque et al., 2009). The above-mentioned effects of endoglin might affect atherosclerosis differently and therefore a precise role of endoglin in this process has not been fully elucidated so far.

More recently, Rossi et al. demonstrated that endothelial endoglin is involved in inflammation and plays a role in leukocyte adhesion and transmigration *in vitro* and *in vivo*. In addition, cell adhesion molecules participated in this process with endoglin, suggesting that endoglin might be considered as a cell adhesion molecule (Rossi et al., 2013).

Both P-selectin and VCAM-1 represent crucial cell adhesion molecules that are the hallmarks of endothelial dysfunction and atherogenesis (Joseph-Silverstein and Silverstein, 1998). Their expression is increased by several stimuli, including cholesterol diet, as demonstrated in rabbits, mice and humans (Davies et al., 1993; Li et al., 1993; Nakashima et al., 1998). Both P-selectin and VCAM-1 are proatherogenic and proinflammatory markers strongly expressed in vascular endothelium in atherosclerosis prone arteries (Cybulsky et al., 2001; Ley and Huo, 2001).

Our previous papers showed endoglin expression exclusively in endothelium of aortic sinus in mice with advanced atherosclerosis (Rathouska et al., 2011; Strasky et al., 2011; Vecerova et al., 2012). However, a detailed immunohistochemical analysis of endoglin expression in aortic sinus and aorta during progression of atherosclerosis has not been reported so far. In addition, a possible expression of endoglin with pro-inflammatory P-selectin and VCAM-1 has not been demonstrated *in vivo* in aorta.

In this study, we set two goals. The first aim was the evaluation of endoglin expression in two different parts of aorta (heart aortic sinus and ascending aorta) in apoE-deficient mice fed either chow or an atherogenic diet (Western type diet). The second aim was the examination of a possible simultaneous expression of endoglin with P-selectin and VCAM-1, in order to evaluate a potential cooperation of endoglin with cell adhesion molecules in apoE-deficient mice during atherogenesis.

Materials and methods

Animals

Animal studies met the accepted criteria for human care and experimental use of laboratory animals. All protocols were approved by the Ethical Committee for the protection of animals against cruelty at Faculty of Pharmacy, Charles University in Prague and all experiments were carried out in accordance with Czech law No. 246/1992.

Ten-week–old female apoE-deficient mice on a C57BL/6J background (n=24) (Taconic, Denmark) were randomly subdivided into three groups.

All mice were fed with two different experimental diets with water *ad libitum* throughout the study. The Chow group of animals (n=8) was fed with chow diet for another two months. There were two Western type diet groups; in the first group (n=8), Western type diet containing 21% fat (11% saturated fat) and 0.15% cholesterol was fed for another two months, in the second group (n=8), the same diet was fed for another four months.

Each mouse, in all 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 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. 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 before immunohistochemical staining. Blood samples were taken from vena cava inferior into heparin-coated tubes and centrifuged at 9000 rpm for 15 min. Collected plasma samples were subsequently stored at -80°C before biochemical analysis.

Biochemical analysis

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: very low-density lipoprotein (VLDL <1.006 g/ml; LDL <1.063 g/ml; high-density lipoprotein (HDL) >1.063 g/ml. The total cholesterol 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).

Immunohistochemistry

Aortic sinus and ascending aortas from three groups of mice (one chow diet group and two Western type diet groups) were taken for analysis. 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. The systematic uniform random sampling was performed for fifty sections from each mouse. Each tenth slide was taken and five slides from each mouse were used for detection of each protein (endoglin, VCAM-1, P-selectin, macrophages). The slides were primarily incubated with 5% non-fat dry milk in phosphate buffered saline (PBS) solution for 30 minutes.

For the detection of endoglin, VCAM-1 and macrophages, the slides were thereafter incubated with primary antibodies for 1 hour at room temperature and were developed with anti-rat ImmPRESSTM (mouse adsorbed) polymerized reporter enzyme staining system (Vector Laboratories, USA) in the presence of 200 µg/ml normal mouse IgG (Dako, Denmark) afterwards. For the detection of P-selectin, the slides were first incubated with anti-avidin and anti-biotin solutions (Vector Laboratories, USA). Afterwards, they were incubated with primary antibody for 1 hour at room temperature and then developed with biotin-conjugated horse anti-goat Ig (dilution 1:400 in BSA) (Vector Laboratories, USA) in the presence of 200 µg/ml normal mouse IgG (Dako, Denmark). In the case of all four antibodies, the antibody reactivity was detected by means of diaminobenzidine tetrahydrochloride substrate (Dako, Denmark). The specificity of the immunostaining was assessed by staining with nonimmune isotype-matched immunoglobulins.

Primary antibodies included the following: monoclonal rat anti-mouse endoglin / CD105 (dilution 1/50 in BSA) and monoclonal rat anti-mouse VCAM-1 / CD106 (dilution 1/100 in BSA), both purchased from BD Pharmingen™, USA. For the staining of macrophages, monoclonal rat anti-mouse macrophages/monocytes antibody (dilution 1/100 in BSA) from AbD Serotec, UK was used. For the staining of P-selectin, polyclonal goat antibody (dilution 1/50 in BSA) directed to P-selectin (Santa Cruz Biotechnology, USA) was used.

Photo documentation and image digitizing from the microscope were performed with the Olympus AX 70, with a digital firewire camera Pixelink PL-A642 (Vitana Corporation, Canada) with image analysis software NIS ver 3.1 (Laboratory Imaging, Prague, Czech Republic).

Plaque size evaluation

Quantitative analysis of plaque size was evaluated by means of orcein-hematoxylin histological staining and stereological methods. Sequential tissue sectioning and systematic uniform random sampling was performed for fifty sections from each mouse as mentioned above. Each sixth slide was used for orcein-hematoxylin staining. Thus, eight sections from each mouse were used for the stereological estimation. Briefly, the staining was performed by incubation of the slides in orcein solution for 30 minutes and thereafter counterstained with hematoxylin for 30 seconds. Photo documentation and image digitizing from the microscope were performed as mentioned above. Stereological analysis was performed with PointGrid module of the ELLIPSE software (ViDiTo, Slovakia) as previously described (Nachtigal et al., 2004).

Results

Biochemical analysis of serum lipid levels in mice

Analysis of blood lipid spectrum in apoE-deficient mice (Fig. 1) revealed significantly higher levels of total cholesterol in mice fed Western type diet for two months (Apo west 2m) in comparison with mice fed chow diet (29.96±2.59 vs. 10.98±0.87, P<0.001). Similarly, comparison of total cholesterol levels between mice fed Western type diet for four months (Apo west 4m) and mice fed chow diet (Apo chow) revealed significantly higher levels in Apo west 4m group (25.60±0.10 vs. 10.98±0.87, P<0.001). There were no significant changes between Apo west 2m and Apo west 4m in total cholesterol levels.

VLDL levels were significantly higher in Apo west 2m group in comparison with Apo chow group (20.55±2.01 vs. 7.98±0.74, P<0.001). Similarly, VLDL levels were significantly higher in Apo west 4m group in comparison with Apo chow group (19.27±0.75 vs. 7.98±0.74, P<0.001). There were no significant changes

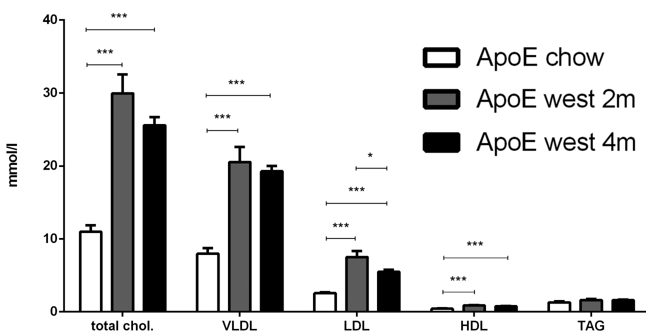

Fig. 1. Blood cholesterol levels in mice on chow diet and Western type diet for two and four months. Values are means ± SEM, n=8. ***P<0.001, *P<0.05.

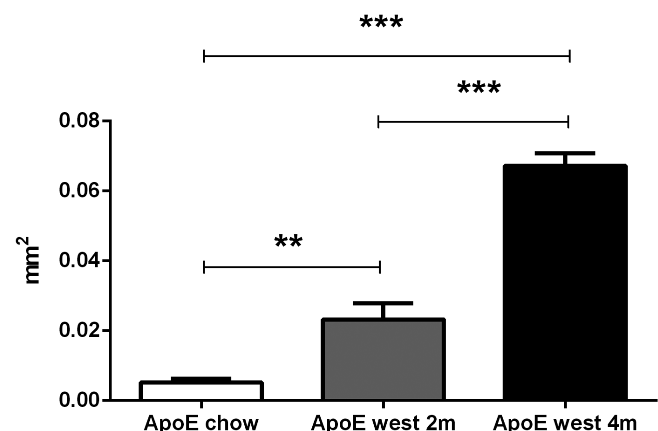

Fig. 2. Stereological analysis of lesion area size in aorta of mice on chow diet and Western type diet for two and four months. Lesion area size continually increased according to diet used and the period of feeding. Values are means ± SEM, n=8. ***P<0.001, **P<0.01.

between Apo west 2m and Apo west 4m in VLDL levels.

LDL levels were significantly higher in Apo west 2m group in comparison with Apo chow group (7.51±0.85 vs. 2.56±0.13, P<0.001). Similarly, LDL levels were significantly higher in Apo west 4m group in comparison with Apo chow group (5.48±0.30 vs. 2.56±0.13, P<0.001). There were also significantly higher levels of LDL in Apo west 2m mice in comparison with Apo west 4m mice (7.51±0.85 vs. 5.48±0.30, P<0.05).

HDL levels were significantly higher in Apo west 2m group in comparison with Apo chow group (0.87±0.07 vs. 0.44±0.04, P<0.001). Similarly, HDL levels were significantly higher in Apo west 4m group in comparison with Apo chow group (0.75±0.06 vs. 0.44±0.04, P<0.001). There were no significant changes between Apo west 2m and Apo west 4m in HDL levels. There were also no significant changes in triglyceride (TAG) levels between any compared groups (Fig. 1).

Atherosclerosis lesion size quantification

The size of atherosclerotic lesions was assessed by stereological analysis of orcein-hematoxylin staining. Administration of Western type diet was associated with a larger plaque size in both Western type diet groups (Apo west 2m, Apo west 4m) in comparison with chow diet group (Apo chow). The plaque size was significantly larger in Apo west 2m group in comparison with Apo chow group (0.023±0.005 vs. 0.005±0.001 mm^2, P<0.01). The plaque size was also significantly larger in Apo west 4m group in comparison with Apo chow group (0.067±0.004 vs. 0.005±0.001 mm^2, P<0.001). The plaque size was even significantly larger in ApoE west 4m group in comparison with ApoE west 2m group (0.067±0.004 vs. 0.023±0.005 mm^2, P<0.001) (Fig. 2).

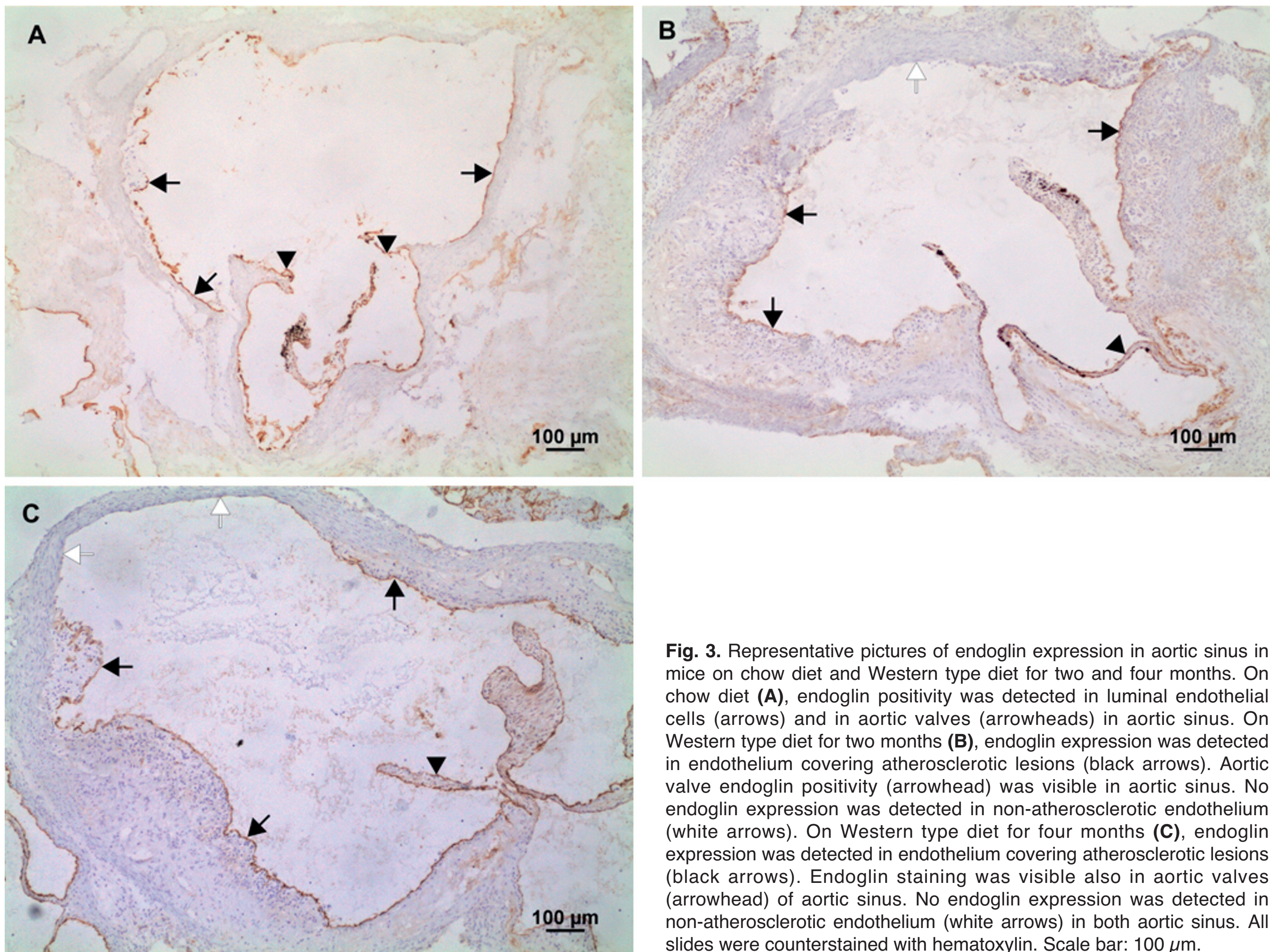

Fig. 3. Representative pictures of endoglin expression in aortic sinus in mice on chow diet and Western type diet for two and four months. On chow diet **(A)**, endoglin positivity was detected in luminal endothelial cells (arrows) and in aortic valves (arrowheads) in aortic sinus. On Western type diet for two months **(B)**, endoglin expression was detected in endothelium covering atherosclerotic lesions (black arrows). Aortic valve endoglin positivity (arrowhead) was visible in aortic sinus. No endoglin expression was detected in non-atherosclerotic endothelium (white arrows). On Western type diet for four months **(C)**, endoglin expression was detected in endothelium covering atherosclerotic lesions (black arrows). Endoglin staining was visible also in aortic valves (arrowhead) of aortic sinus. No endoglin expression was detected in non-atherosclerotic endothelium (white arrows) in both aortic sinus. All slides were counterstained with hematoxylin. Scale bar: 100 μm.

Endoglin expression in mice on chow diet and on Western type diet for two and four months

Immunohistochemical analysis of endoglin expression was performed in the aortic sinus inside the heart and in the ascending part of aorta in all studied animals.

In general, endoglin expression was detected only in endothelial cells in all studied animals. Endoglin positivity was visible in myocardial capillaries and in endocardial simple squamous epithelial cells (data not shown). Endoglin expression in aorta was detected only in luminal endothelial cells but not in the vessel media.

In mice on chow diet, endoglin expression covered almost the whole luminal area, including non-atherosclerotic endothelium and endothelium covering small atherosclerotic lesions in aortic sinus. Moreover, endoglin positivity was also detected in aortic valves of the aortic sinus (Fig. 3A). On the contrary, almost no endoglin expression was detected in aortic endothelium in ascending aorta, where no atherosclerotic lesions were present (Fig. 4A).

Atherosclerotic lesions were visible in both aortic sinus and ascending aorta in mice fed Western type diet for two and four months.

In mice fed Western type diet for two months, endoglin expression was detected almost exclusively in aortic endothelium covering atherosclerotic lesions but not in non-atherosclerotic endothelium in aortic sinus (Fig. 3B). Moreover, endoglin expression was also detected in myocardial capillaries and aortic valves (Fig. 3B). Similarly, like in mice on chow diet, endoglin expression was weaker in aorta when compared to aortic sinus. In addition, endoglin was detected almost exclusively in endothelium covering atherosclerotic plaques but not in non-atherosclerotic endothelium in ascending aorta (Fig. 5A). In some parts of vessels, where no atherosclerotic lesions were visible, no

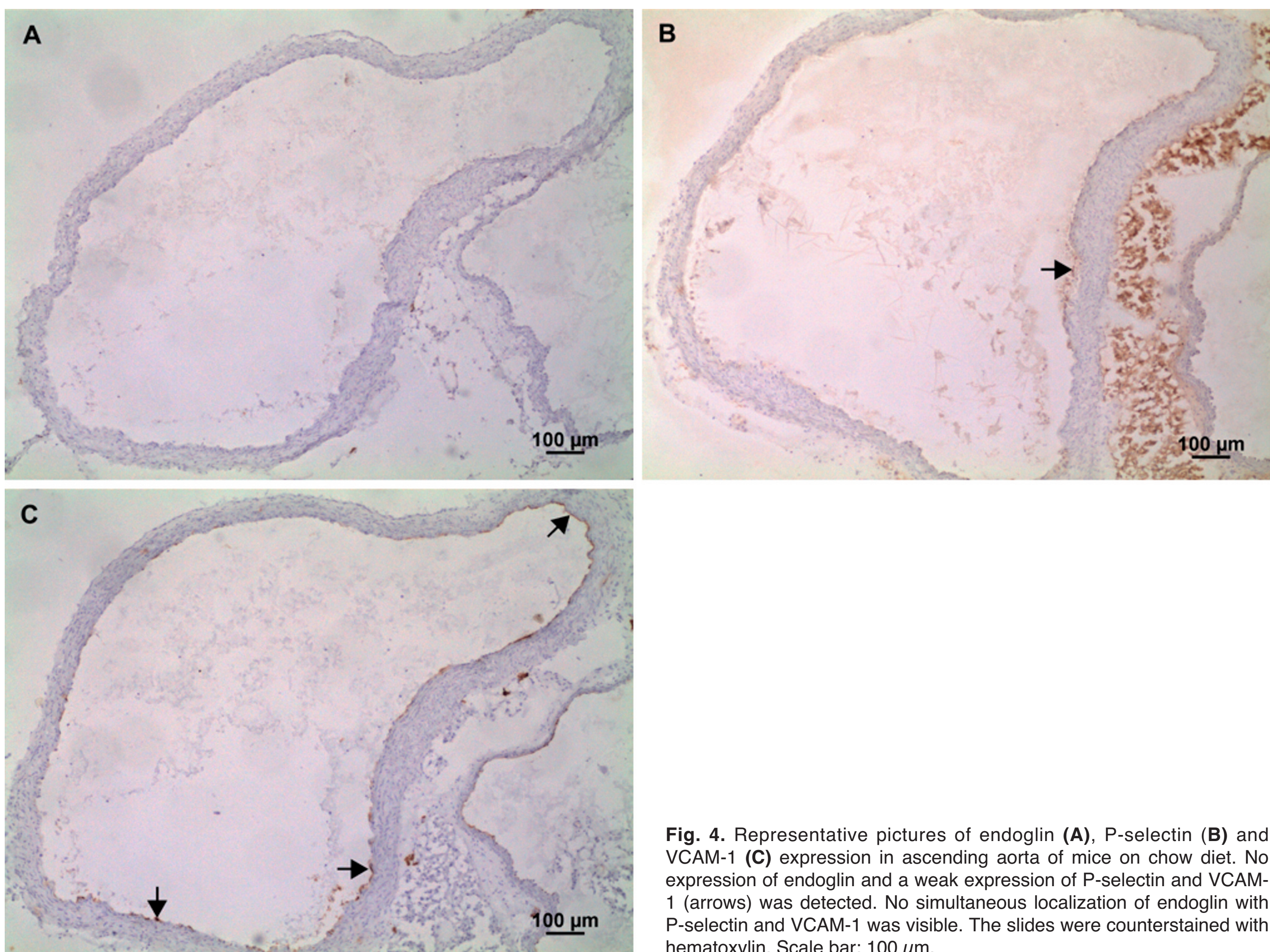

Fig. 4. Representative pictures of endoglin **(A)**, P-selectin (**B)** and VCAM-1 **(C)** expression in ascending aorta of mice on chow diet. No expression of endoglin and a weak expression of P-selectin and VCAM-1 (arrows) was detected. No simultaneous localization of endoglin with P-selectin and VCAM-1 was visible. The slides were counterstained with hematoxylin. Scale bar: 100 μm.

endoglin expression was detected (data not shown).

Similar staining patterns of endoglin expression were visible in mice on Western type diet for four months. Endoglin expression was detected almost exclusively in aortic endothelium covering atherosclerotic lesions but not in non-atherosclerotic endothelium in aortic sinus (Fig. 3C). In addition, endoglin staining was visible in aortic valves of aortic sinus (Fig. 3C). Endoglin expression was also detected almost exclusively in aortic endothelium covering atherosclerotic lesions but not in non-atherosclerotic endothelium in ascending aorta (Fig. 6A). In areas with no atherosclerotic lesions, no endoglin expression was visible (data not shown). Endoglin expression seemed to be weaker in aorta when compared to aortic sinus.

Analysis of simultaneous endoglin expression with P-selectin and VCAM-1 in ascending aorta

Since endoglin expression did not reflect the atherogenic process in aortic sinus, we decided to evaluate its expression with P-selectin and VCAM-1 only in ascending aorta, where changes of endoglin expression in the course of atherogenesis were detected. In general, P-selectin expression was detected only in endothelium, while VCAM-1 was also visible inside atherosclerotic lesions and in vessel media in ascending aorta.

A weak P-selectin and a stronger VCAM-1 expression was detected only in aortic endothelium in mice on chow diet. No endoglin expression in these vessels means no simultaneous localization of endoglin, P-selectin and VCAM-1 in mice on chow diet (Fig. 4A-C).

A strong endoglin endothelial staining and a substantially weaker P-selectin endothelial expression was detected in mice fed Western type diet for two months (Fig. 5A,B, respectively). VCAM-1 expression was visible inside the atherosclerotic lesions and in aortic media mostly underneath the atherosclerotic

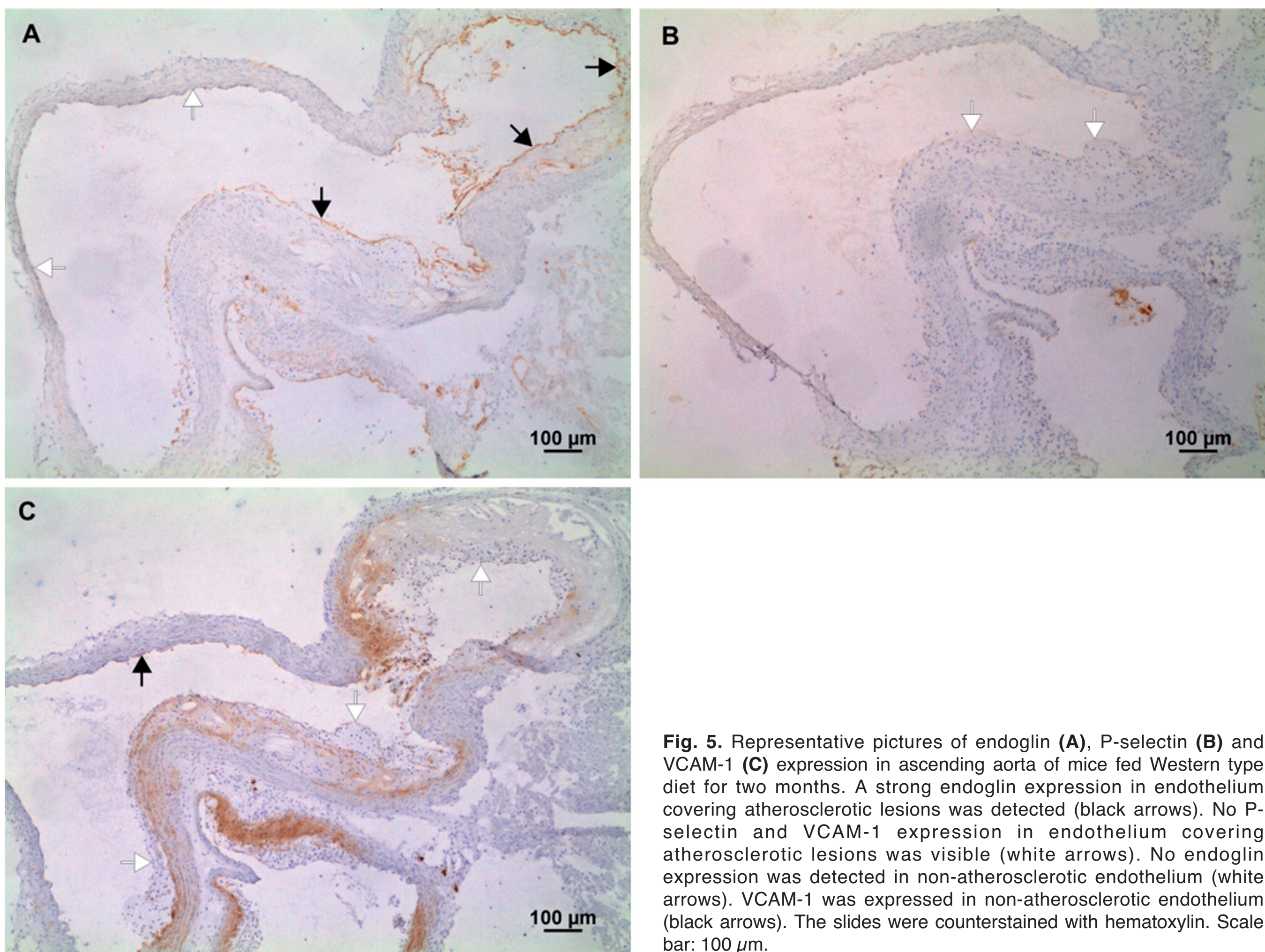

Fig. 5. Representative pictures of endoglin **(A)**, P-selectin **(B)** and VCAM-1 **(C)** expression in ascending aorta of mice fed Western type diet for two months. A strong endoglin expression in endothelium covering atherosclerotic lesions was detected (black arrows). No P-selectin and VCAM-1 expression in endothelium covering atherosclerotic lesions was visible (white arrows). No endoglin expression was detected in non-atherosclerotic endothelium (white arrows). VCAM-1 was expressed in non-atherosclerotic endothelium (black arrows). The slides were counterstained with hematoxylin. Scale bar: 100 μm.

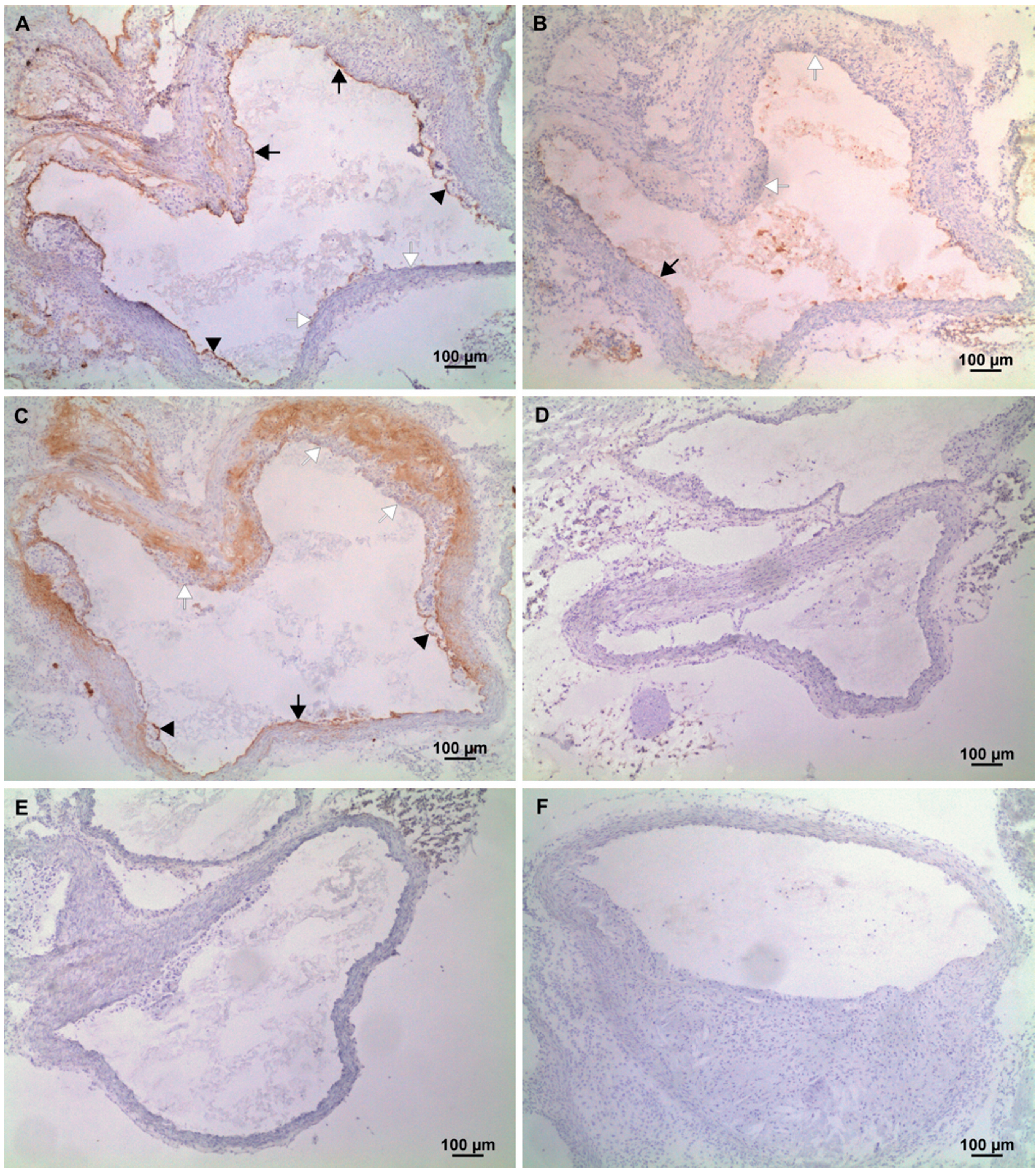

Fig. 6. Representative pictures of endoglin **(A)**, P-selectin **(B)** and VCAM-1 **(C)** expression in ascending aorta in mice fed Western type diet for four months. A strong endoglin expression in endothelium covering atherosclerotic lesions was detected (black arrows). No P-selectin and VCAM-1 expression in endothelium covering atherosclerotic lesions was visible (white arrows). No endoglin expression was detected in non-atherosclerotic endothelium (white arrows). Occasional VCAM-1 expression with endoglin was visible in small fatty streaks (black arrowheads). VCAM-1 and P-selectin were slightly expressed in non-atherosclerotic endothelium (black arrows). Specificity of the immunostaining was assessed by omitting of primary antibody and staining with nonimmune isotype-matched immunoglobulins for endoglin **(D)**, P-selectin **(E)** and VCAM-1 **(F)**. The slides were counterstained with hematoxylin. Scale bar: 100 μm.

lesions. Moreover, VCAM-1 expression was detected in non-atherosclerotic endothelium and weakly in some vessels in endothelium covering atherosclerotic lesions (Fig. 5C). No substantial localization of endoglin with P-selectin and VCAM-1 was detected in mice fed Western type diet for two months (Fig. 5A-C).

Similar staining patterns of endoglin, P-selectin and VCAM-1 expression were demonstrated in mice aorta of mice fed Western type diet for four months (Fig. 6A-C). Endoglin expression was visible only in endothelium covering atherosclerotic plaques, P-selectin expression was hardly detected and VCAM-1 expression was visible inside the atherosclerotic lesions, in aortic media in non-atherosclerotic endothelium and in some vessels in endothelium covering small fatty streaks. A weak simultaneous expression of endoglin and VCAM-1 but not with P-selectin was visible only in some vessels in endothelium covering small fatty streaks (Fig. 6A-C).

Macrophages staining in aorta of apoE-deficient mice

Immunohistochemical staining showed an accumulation of macrophages during the atherogenic process. No or a weak positivity for macrophages accumulated in aortic intima was visible in most of the vessels in mice on chow diet (Fig. 7A). On the other hand, the accumulation of macrophages in atherosclerotic lesions was visible in mice fed Western type diet for two and four months (Fig. 7B,C). Macrophage accumulation was stronger in mice fed Western type diet for four months when compared to mice fed the diet for two months (Fig. 7B,C).

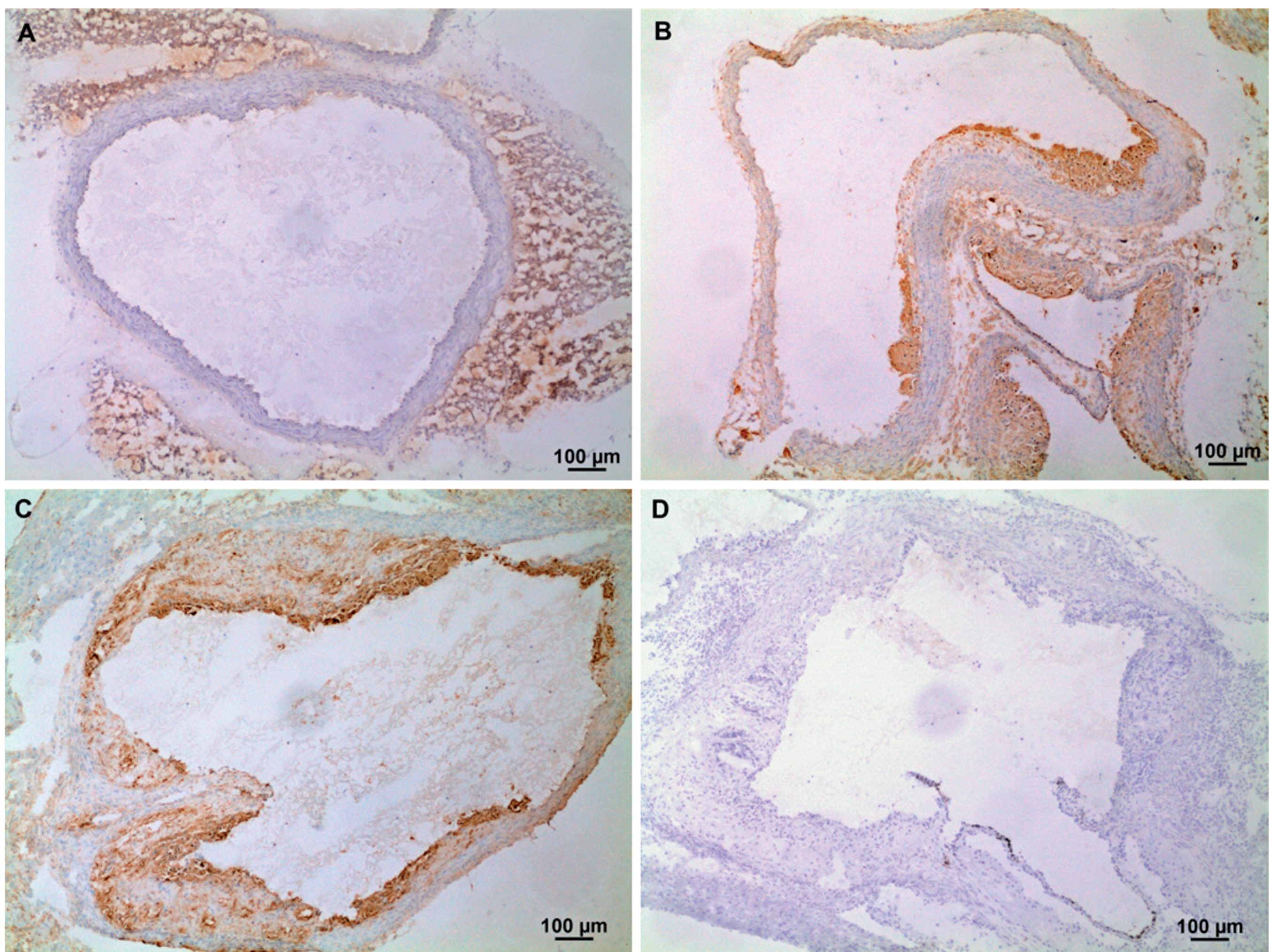

Fig. 7. Representative pictures of macrophage staining in mice on chow diet **(A)** and Western type diet for two **(B)** and four **(C)** months. Macrophage accumulation in intima was barely detectable in mice on chow diet but it was clearly visible in mice fed Western type diet for two and four months. Specificity of the immunostaining was assessed by omitting of primary antibody and staining with nonimmune isotype-matched immunoglobulins for macrophages **(D)**. The slides were counterstained with hematoxylin. Scale bar: 100 μm.

Discussion

Endoglin expression in atherosclerosis has been studied in both humans and experimental animals. In general, endoglin expression was detected in cells that are important for atherogenesis and vascular homeostasis, including endothelial cells, monocyte/macrophages, as well as in vascular smooth muscle cells (Nachtigal et al., 2012). However, controversial data come from these studies with respect to endoglin localization in atherosclerotic vessels and its possible role in atherogenesis.

Therefore, the first aim of this study was to evaluate changes of endoglin localization in the part of aorta located inside the heart (aortic sinus) and in ascending aorta located outside the heart. Secondly, we wanted to evaluate a possible simultaneous expression of endoglin with cell adhesion molecules involved in atherogenesis (P-selectin, VCAM-1).

For that purpose we used apoE-deficient mice fed chow diet, Western type diet (two months) and Western type diet (four months) in order to see possible changes of endoglin expression during different phases of the atherogenic process. Indeed, a cholesterol diet significantly increased the levels of cholesterol, increased the atherosclerotic plaque size and resulted in macrophage accumulation in aortic plaques. These changes in the aorta allowed us to study endoglin expression changes with respect to development of atherosclerosis in aorta.

Most of the studies published in humans showed endoglin expression almost exclusively in endothelium of microvessels. Li et al. showed a weak or no expression of endoglin in normal arteries but a strong expression in atherosclerotic carotid arteries, predominantly in microvessels of the lipid core (Li et al., 2006). Additionally, endoglin expression was strong in neovessels within the carotid plaque, predominantly within vulnerable plaque shoulders, and within the lipid core (Luque et al., 2008). In another study, endoglin expression was strongest in advanced lesions in neovessels in the human carotid and coronary arteries when compared with early lesions (Luque et al., 2009). More recently, Li et al. showed that endoglin expression in coronary artery microvessels correlated with plaque hemorrhages (Li et al., 2012).

These studies suggested a possible participation of endoglin in plaque neoangiogenesis and in the regulation of intraplaque inflammation, because these plaque microvessels can serve as a gateway for the recruitment of inflammatory cells into atherosclerotic plaques (de Boer et al., 1999). On the contrary, another study also showed endoglin expression in carotid plaque neovessels, although these authors suggested that endoglin together with other markers might be important for the formation of neovessels less prone to leakage, rupture and hemorrhage (Slevin et al., 2010). Surprisingly, there was no information about endoglin expression in smooth muscle cells, macrophages or luminal endothelial cells in these studies.

On the other hand, some other papers showed different endoglin expression in non-atherosclerotic and atherosclerotic vessels. Conley et al. detected a weak expression of endoglin in non-atherosclerotic aortas and carotid arteries but a strong endoglin expression in advanced atherosclerotic plaques in smooth muscle cells, again with no positivity in luminal endothelial cells or macrophages (Conley et al., 2000). Piao et al. demonstrated a weak endoglin expression in normal arteries but a higher expression of endoglin in macrophages, smooth muscle cells and endothelial cells in early lesions when compared with advanced lesions (Piao and Tokunaga, 2006). These papers suggested that endoglin participates in vascular repair and possibly in atherogenesis.

Surprisingly, Bot et al. focused on the expression of endoglin in human carotid endarterectomy showing endoglin expression in intraplaque vessels (endothelial cells), macrophages and smooth muscle cells. Moreover, endoglin expression was higher in plaques containing higher levels of collagen and less thrombi in the plaque, suggesting that endoglin expression is related to a more stable plaque phenotype (Bot et al., 2009).

These discrepancies in human studies might be related to the fact that different vessels in different stages of atherosclerosis were studied and we cannot rule out the possibility of different reactions of various antibodies used in these studies.

It is of interest to mention that no human study focusing on the expression of endoglin during atherogenesis mentioned endoglin expression in aortic, carotid or coronary luminal endothelial cells and there has also been no data from experimental studies in mice concerning endoglin expression in these parts of the vascular tree so far.

We showed endoglin expression in endothelial cells in myocardial capillaries and luminal endothelial cells in aortic sinus and aorta of apoE-deficient mice. In addition, endoglin expression was also detected in aortic valves and simple squamous epithelium in endocardium in this study. This is in line with previously published papers showing that endoglin is not expressed by smooth muscle and macrophages in mice atherosclerosis (Nachtigal et al., 2009b; Rathouska et al., 2011; Strasky et al., 2011; Vecerova et al., 2012). On the other hand, no neovessels were visible in our mice. Endoglin expression in luminal endothelial cells was very strong inside the heart in aortic sinus. In fact, almost all luminal endothelial cells were stained for endoglin, regardless of the presence or stage of atherosclerotic process. Considering endoglin expression in aortic valves and endocardium, we might propose that endoglin expression in aortic sinus is not related to the progression of atherosclerosis/atherogenesis but might more likely be related to the role of endoglin in heart and valve development as demonstrated previously (Qu et al., 1998).

On the other hand, a different reaction was observed

in the aorta outside the heart. In general, endoglin positivity was weaker in this part of aorta when compared to aortic sinus in all studied groups. In addition, endoglin expression was almost absent in aortic endothelium in vessels or parts of vessels where no atherosclerotic lesions were found, irrespective of the diet. In other words, concerning the parts of aorta with no relation to heart and valve development, endoglin expression was detected only in endothelial cells on the surface of atherosclerotic lesions in all mouse groups. Our previous papers showed endoglin co-localization with eNOS in mice atherosclerosis (Nachtigal et al., 2009a,b; Vecerova et al., 2012). In addition, endoglin is able to affect expression and activity of eNOS (Jerkic et al., 2004; Toporsian et al., 2005; Cudmore et al., 2007). We might speculate that endoglin expressed only by plaque endothelium might be related to its endothelial protective role together with eNOS. On the other hand, Rossi et al. demonstrated recently that endoglin might be involved in leukocyte adhesion during transmigration both *in vitro* and *in vivo* and cooperates in this process with cell adhesion molecules (Rossi et al., 2013). Despite very convincing data from the paper, it is necessary to point out that endoglin role in leukocyte adhesion was demonstrated in venules in the study. Indeed, venules are critical vessels for inflammatory reaction in most organs (Scalia, 2013). However, homeostasis of arteries as vessels prone to the development of atherosclerosis is different when compared to venules or capillaries (different size, structure and hemodynamic conditions).

VCAM-1 and P-selectin are critical cell adhesion molecules participating in the development of endothelial dysfunction and atherogenesis (Ramos et al., 1999; Ley and Huo, 2001). Early P-selectin and VCAM-1 endothelial expression was demonstrated even before the formation of atherosclerotic lesions (Li et al., 1993), suggesting that they are markers of endothelial activation. Because of the fact that endoglin reflected the atherogenic process only in ascending aorta in this study, we focused on endoglin expression with P-selectin and VCAM-1 only in this part of the vessel. Weak P-selectin and VCAM-1 expressions were detected in non-atherosclerotic endothelium, although no simultaneous expression with endoglin was observed, suggesting that endoglin is not involved in early activation of endothelium in mice atherogenesis. In addition, no simultaneous expression of endoglin with P-selectin and only a weak occasional expression of VCAM-1 and endoglin was visible in endothelium covering atherosclerotic plaques. Based on the results of this immunohistochemical study focusing on mice aorta with different phases of atherogenesis, we might propose that endoglin is not expressed together with cell adhesion molecules that are critical for inflammation and atherogenesis. These results, of course, do not rule out a possibility that endoglin participates in the inflammation and leukocyte adhesion in other parts of the vascular tree or other organs. It must be pointed out that endoglin plays different roles in various organs and in various pathological conditions. Endoglin related activation and overexpression of eNOS might be related to the improvement of endothelial dysfunction (Jerkic et al., 2004) (antiatherogenic effect) or activation of angiogenesis (pro-atherogenic) (Duda et al., 2004; Li et al., 2012). In addition, endoglin related increase of collagen production might increase the stability of atherosclerotic plaques (Bot et al., 2009) or support a cardiac fibrosis (Kapur et al., 2012).

It must be stated that immunohistochemical analysis used in this study cannot answer the question about the proatherogenic or antiatherogenic role of endoglin in atherosclerosis; however, it brings another piece of the puzzle with respect to evaluation the role of endoglin in atherogenesis.

In summary, we showed that: 1) endoglin expression is detected in endothelial cells but not in macrophages or smooth muscle cells in aortic sinus and aorta in apoE-deficient mice, 2) endoglin expression varies during the atherogenic process in aorta but not in aortic sinus, 3) endoglin expression is detected only in endothelium covering atherosclerotic lesions but not in non-atherosclerotic endothelium regardless of the plaque size and 4) endoglin is not expressed together with P-selectin and VCAM-1 in aortic endothelium.

In conclusion, this study shows that endothelial expression of endoglin is related to the atherogenic process predominantly in aorta outside the heart. Moreover, endoglin is not localized with cell adhesion molecules involved in atherosclerosis, suggesting it might not participate in leukocyte accumulation in aorta of apoE-deficient mice during atherogenesis.

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RESEARCH ARTICLE

High Soluble Endoglin Levels Do Not Induce Endothelial Dysfunction in Mouse Aorta

Ivana Nemeckova[1☯], Agnieszka Serwadczak[2☯], Barbara Oujo[4☯], Katerina Jezkova[1], Jana Rathouska[1], Petra Fikrova[1], Michala Varejckova[1], Carmelo Bernabeu[5], Jose M. Lopez-Novoa[4], Stefan Chlopicki[2,3], Petr Nachtigal[1]*

1 Department of Biological and Medical Sciences, Faculty of Pharmacy in Hradec Kralove, Charles University in Prague, Heyrovskeho 1203, Hradec Kralove, 500 05, Czech Republic, **2** Jagiellonian Centre for Experimental Therapeutics (JCET), Bobrzynskiego 14, 30-348, Krakow, Poland, **3** Department of Experimental Pharmacology, Chair of Pharmacology, Medical College Jagiellonian University, Grzegorzecka 16, 31–531 Krakow, Poland, **4** Renal and Cardiovascular Physiopathology Unit, Department of Physiology and Pharmacology, University of Salamanca, 37007 Salamanca, Spain, **5** Centro de Investigaciones Biologicas, Consejo Superior de Investigaciones Cientificas, CSIC, and Centro de Investigacion Biomedica en Red de Enfermedades Raras (CIBERER), 28040 Madrid, Spain

☯ These authors contributed equally to this work.
* petr.nachtigal@faf.cuni.cz

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Abstract

Increased levels of a soluble form of endoglin (sEng) circulating in plasma have been detected in various pathological conditions related to cardiovascular system. High concentration of sEng was also proposed to contribute to the development of endothelial dysfunction, but there is no direct evidence to support this hypothesis. Therefore, in the present work we analyzed whether high sEng levels induce endothelial dysfunction in aorta by using transgenic mice with high expression of human sEng. Transgenic mice with high expression of human sEng on CBAxC57Bl/6J background (*Sol-Eng*$^+$) and age-matched transgenic littermates that do not develop high levels of human soluble endoglin (control animals in this study) on chow diet were used. As expected, male and female *Sol-Eng*$^+$ transgenic mice showed higher levels of plasma concentrations of human sEng as well as increased blood arterial pressure, as compared to control animals. Functional analysis either *in vivo* or *ex vivo* in isolated aorta demonstrated that the endothelium-dependent vascular function was similar in *Sol-Eng*$^+$ and control mice. In addition, Western blot analysis showed no differences between *Sol-Eng*$^+$ and control mice in the protein expression levels of endoglin, endothelial NO-synthase (eNOS) and pro-inflammatory ICAM-1 and VCAM-1 from aorta. Our results demonstrate that high levels of soluble endoglin alone do not induce endothelial dysfunction in *Sol-Eng*$^+$ mice. However, these data do not rule out the possibility that soluble endoglin might contribute to alteration of endothelial function in combination with other risk factors related to cardiovascular disorders.

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INTRODUCTION

Endoglin/CD105 (Eng) is an accessory type III receptor for several members of the transforming growth factor-β (TGF-β) superfamily of proteins. This homodimeric, 180 kDa, transmembrane glycoprotein is considered to play an eminent role in hematopoiesis, angiogenesis, cardiovascular development and vascular tone [1]. Endoglin is expressed predominantly in endothelial cells, but it can also be detected in various other cells, including smooth muscle cells, mesenchymal and hematopoietic stem cells, monocytes/macrophages, placental syncytiotrophoblasts and fibroblasts [2].

In addition to membrane-bound endoglin, increased levels of a soluble form of endoglin (sEng) have been detected in plasma in various pathological conditions related to the cardiovascular system. Circulating sEng represents the NH_2-terminal cleavage product of full-length membrane-bound endoglin [3] and was proposed to be cleaved from the intact membrane form of several cell types including endothelial cells and trophoblasts by matrix metalloproteinase-14 (MMP-14 or MT-1) [4,5].

There is a number of reports suggesting that soluble endoglin may be regarded as a biomarker of endothelial dysfunction, for example in pre-eclampsia [3], atherosclerosis [6,7], hypercholesterolemia [8], diabetes mellitus and hypertension [9] and chronic coronary artery disease [10]. Because in all of these pathologies the endothelial dysfunction plays an important role, it was also proposed that high levels of soluble endoglin might represent a hallmark of endothelial dysfunction contributing to the development of numerous cardiovascular diseases, including pre-eclampsia and atherosclerosis [11]. On the other hand, it was also demonstrated that soluble endoglin is able to modify TGF-β-dependent signaling in vascular endothelium [12]. Despite being related to several cardiovascular pathologies, it is still unclear whether soluble endoglin represents a mere biomarker or it is mechanistically involved in vascular pathology via e.g. induction of endothelial dysfunction.

The increased expression of cell adhesion molecules, the impairment of NO bioavailability and NO-dependent vasodilatation are the general hallmarks of endothelial dysfunction, the crucial step in the pathogenesis of atherosclerosis [13–15]. Interestingly, it was demonstrated that soluble endoglin increases the expression of cell adhesion molecules, the number of rolling leukocytes and impairs endothelial dependent vascular function [12]. Furthermore, we have recently observed that soluble endoglin impairs leukocyte rolling and binding to endothelium *"in vitro"* [16].

In order to study the pathogenic role of soluble circulating form of Eng, a transgenic mouse model expressing human soluble endoglin (*Sol-Eng*$^+$) has been recently generated. The *Sol-Eng*$^+$ mice exhibit a pre-eclampsia-like phenotype, including hypertension, small pup size, proteinuria and renal damage [5].

Considering a possible role of soluble endoglin in the development of systemic endothelial dysfunction, in the present work, we have assessed whether in *Sol-Eng*$^+$ mice the endothelial dysfunction in aorta can be detected, as compared to their transgenic littermates with low levels of soluble endoglin.

MATERIALS AND METHODS

Animals and study design

A mouse line that overexpresses human soluble endoglin (*Sol-Eng*$^+$) on the CBAxC57BL/6J background was generated at the Genetically Modified Organisms Generation Unit (University of Salamanca, Spain), as previously described [5]. Four to six month old *Sol-Eng*$^+$ male and female mice with high plasma levels of soluble endoglin and their age matched male and female

littermates with low plasma levels of soluble endoglin (transgenic mice with low plasma levels of sEng used as control mice) were used. The animals were housed under a 12-h light cycle and constant temperature and humidity and had free access to tap water and a standard laboratory pellet diet.

All experiments were performed in accordance with the directive of the EEC (86/609/EEC) and the use of animals was approved by the Ethical Committee for the protection of animals against cruelty at Faculty of Pharmacy in Hradec Kralove, Charles University in Prague (Permit Number: 21558/2013–2), and the Bioethics Committee of the University of Salamanca (Permit Number: 006–201400038812).

All surgery procedures were carried out under ketamine/diazepam/atropine or ketamine/xylazine anesthesia, and all efforts were made to minimize the suffering of the animals.

Analysis of soluble endoglin concentration in plasma

Blood was extracted using a tail tip and plasma levels of human soluble endoglin were determined by means of Human Endoglin/CD105 Quantikine ELISA Kit (R&D Systems, MN, USA) according to the instructions of the manufacturer.

Blood Pressure and heart rate measurement

Basal Blood pressure (BP) was recorded in conscious mice with an automated multichannel system by using the tail-cuff method and a photoelectric sensor (Niprem 546; Cibertec, Spain). Animals were previously accustomed for several days and measures were collected at the same hour during at least 3 days, as previously described [17–19]. Acute changes in BP and heart rate (HR) after drug administration were measured in conscious, freely moving mice by radiotelemetry techniques as previously described [17]. In brief, after anesthesia of the animals with a mixture of ketamine 78 mg/Kg, diazepam 6 mg/Kg, and atropine 0.15 mg/Kg; i.p., the carotid artery of the mouse was accessed with a ventral midline incision, and cannulated with a catheter attached to a combination pressure transducer, transmitter and battery, all encapsulated in an implantable microminiaturized electronic monitor (PA-C20, Data Sciences International-DSI-; MN, USA). The skin was closed with staples and tissue adhesive, and topical antiseptic was applied. An analgesic, (buprenorphine 0.1 mg/Kg i.m., Buprex, Schering-Plough, Spain) was given at the end of the surgery. An antibiotic (cefazolin 25 mg/Kg, i.m., Normon, Spain) was administered at the time of the operation and twice daily during recovery. Approximately 1 mL of normal saline was subcutaneously injected into two or more abdominal sites to assure adequate postoperative hydration, and the animal was kept in a ventilated and warm environment for at least 24 h. Each animal was housed individually in a standard polypropylene cage in a 12:12-h light-dark cycle room, fed standard rodent chow, and given drinking water *ad libitum*. At least 13 days after recovery from the surgical procedures, the cage was placed over a radio receiver in a quiet environment, and repeated measurements of basal systolic (SAP) and diastolic (DAP) arterial pressure and HR were performed in each animal between 10:00 a.m. and 14:00 p.m., for at least 3 days, to ensure stable pressure record. Data was digitally recorded on a computer and results were calculated using the software provided by Data Sciences. To assess the acute effects of the substances to be tested, basal SAP and DAP and HR were recorded for 5 min. Then, animals were given the different substances by i.p. injection in 0.1 mL isotonic NaCl, and AP and HR were continuously recorded for 30 min. Injection of 0.1 mL of isotonic NaCl induced a transient increase in AP and HR that disappeared in 1–2 min and was similar in WT and *Sol-Eng*$^{+}$ mice; thus, indicating that the effects observed for the different substances are not due to animal manipulation or differential response to stress between mouse strains.

Substances tested were: acetylcholine 1 μg/Kg, sodium nitroprusside (SNP) 2 mg/Kg, and the nitric oxide synthase inhibitor—L-NG-nitroarginine methyl ester (L-NAME) 50 mg/Kg.

Analysis of vascular function in isolated mice aorta

Animals received an anesthetic (mixture of ketamine 100 mg/Kg and xylazine 16 mg/Kg; i.p.) overdose and the thoracic part of the aorta was quickly removed, washed in Krebs-Hanseleit buffer and carefully dissected from surrounding tissue. Isolated aorta was cut into 4 rings (each 3 mm long) and placed in organ chambers of the wire myograph (620M, Danish Myo Technology, Denmark). 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% O_2 and 5% CO_2. After an equilibration and heating (37°C) period of 30 min, the tension was stepwise increased to 10 mN for further stabilization for 30 min. The viability of the vessels was checked by KCl (30–60 mM). Aortic rings were pre-contracted with increasing concentrations of phenylephrine (PHE, 0.01–1 μM) or prostaglandin F2alpha (PGF2α, 0.1–10 μM), respectively, to obtain approximately 80% of KCl induced contraction. The endothelium-dependent relaxation was induced by cumulative concentrations of acetylcholine (Ach, 0.01–1 μM). Modulatory effect of NO production on contractility was determined by analyzing the PHE induced contractility before and after the administration of L-NAME (300 μM, incubation 20 min).

Expression of endothelial dysfunction markers in isolated mice aorta by Western blot

Samples of aorta (n = 7 controls, n = 6 *Sol-Eng*$^+$) were homogenized in RIPA lysis buffer (Sigma-Aldrich, St. Louis, USA) with protease (SERVA Electrophoresis, Germany) and phosphatase inhibitors (Thermo Fisher Scientific Inc., IL, USA). Proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and electrically transferred onto PVDF membrane (Milipore, NY, USA) using Trans-Blot SD Semi-Dry Electrophoretic Transfer Cell (Bio-Rad, CA, USA). The membranes were blocked for 1 hour with 5% non-fat dry milk in Tris buffered saline containing 0.1% Tween-20 at room temperature, and then incubated with following primary antibodies: goat polyclonal anti-endoglin (90–95 kDa; dilution 1:500; sc-19793, Santa Cruz Biotechnology, Inc., CA, USA), rabbit polyclonal anti-eNOS (140kDa; dilution 1:200; sc-654, Santa Cruz Biotechnology, Inc.), rabbit polyclonal anti-ICAM-1 (85–110 kDa; dilution 1:500; sc-1511-R, Santa Cruz Biotechnology, Inc.), goat polyclonal anti-VCAM-1 (140 kDa, dilution 1:500; AF643, R&D Systems). Equal loading of proteins onto the gel was confirmed by immunodetection of mouse monoclonal anti-GAPDH antibody (37 kDa; dilution 1:10,000; G8795, Sigma-Aldrich). As secondary antibodies HRP-conjugated goat anti-mouse IgG at 1:20,000 (A9917, Sigma-Aldrich), HRP-linked goat anti-rabbit IgG—(Fab)´2 at 1:2,000 (ab6112, Abcam, UK) and HRP-conjugated rabbit anti-goat IgG at 1:5,000 (A5420, Sigma-Aldrich) were used. Membranes were developed using enhanced chemiluminescent reagent (Thermo Fisher Scientific Inc.) and subsequently exposed to X-Ray films (Foma, Czech Republic). The immunoreactive bands were scanned by using an Epson Perfection V5000 Photo (EPSON Inc., CA, USA) and semiquantified using NIS-Elements software, version 4.0 (Laboratory Imaging, Czech Republic).

Urinary nitrite excretion

Urine was obtained from individual mice housed in metabolic cages for 24 h. Urine was collected in graduated cylinders containing 100 μL of 0.1% sodium azide (to minimize bacterial growth) and 1 mL of mineral oil (to avoid evaporation). Urinary nitrite concentration was

determined in plasma and urine by a modification of the Griess reaction, as described [20]. Briefly, 500 μL of sample were mixed with 250 μL of Griess reagent (1% sulfanilamide and 0.1% naphthyl ethylenediamine dihydrochloride, in 2.5% orthophosphoric acid; Sigma Aldrich) and incubated for 15 min at room temperature. Absorbance was measured at 560 nm and calibration was carried out using sodium nitrite. Urine creatinine concentrations were determined by a modification of Jaffé's reaction method.

Statistical analysis

The statistical analysis was performed by GraphPad Prism 6.0 software (GraphPad Software, Inc., CA, USA). All data are presented as mean ± S.E.M. Direct group-group comparisons were carried out using one or two way ANOVA, paired or unpaired t-test and Mann-Whitney test as adequate. P values of 0.05 or less were considered statistically significant.

RESULTS

Elevated levels of human soluble endoglin in plasma in *Sol-Eng*$^+$ mice

ELISA analysis was used to assess human soluble endoglin levels in studied mice. As shown in Fig. 1, soluble endoglin concentration in plasma was substantially higher in both female (2.477 ± 110.3 vs. 54.68 ± 16.64 ng/ml, respectively) and male (2.579 ± 115.2 vs. 37.79 ± 13.95 ng/mL, respectively) *Sol-Eng*$^+$ mice when compared to control mice (Fig. 1).

Increased arterial pressure in *Sol-Eng*$^+$ mice

Measurements of AP by tail-cuff method (Table 1) show that systolic pressure in male and female *Sol-Eng*$^+$ mice is higher than that of control mice. Arterial pressure was also measured by telemetry, showing that systolic and diastolic AP in *Sol-Eng*$^+$ male mice was higher than that in control littermates (Fig. 2A). In the same group of animals, no significant differences in HR were observed between transgenic and control mice (Fig. 2B).

Preserved NO-dependent vascular response in vivo in *Sol-Eng*$^+$ mice

To test the NO-dependent function *in vivo*, we determined the response of BP to the blockade of nitric oxide (NO) synthesis and nitrite concentration in the urine. Acute administration of the NOS inhibitor L-NAME induced a sustained and similar hypertensive response in both groups of animals (Fig. 3B). Furthermore, acute administration of Ach seems to induce a similar drop of BP in *Sol-Eng*$^+$ mice and control animals (data not shown). However, it should be noted that the effect of acetylcholine with this experimental design was very variable, probably explained because it is very short and in many cases the hypotensive effect was artifacted by the removal of the mice from their cages to inject the drug and the consequent effects on arterial pressure, thus making difficult to correctly assess the hypotensive effect of ACh. Acute administration of sodium nitroprusside (SNP) also induced a significantly fall of BP in *Sol-Eng*$^+$ and control mice (Fig. 3A), with no significant differences between both groups of mice. Urinary excretion of nitrites, a stable-end product of NO metabolism, was also not statistically different in *Sol-Eng*$^+$ *vs*. control mice (Fig. 4).

Preserved NO-dependent vasodilation in *ex vivo* aortic rings in *Sol-Eng*$^+$ mice

Endothelium-dependent vasodilation induced by acetylcholine (Ach) in PHE or PGF2α (1 μM) pre-contracted aorta was similar in *Sol-Eng*$^+$ and control female mice and also in *Sol-Eng*$^+$ and control male animals (81.18±3.26 *vs*. 73.65±2.46, 85.01±3.13 *vs*. 78.9±6.26,

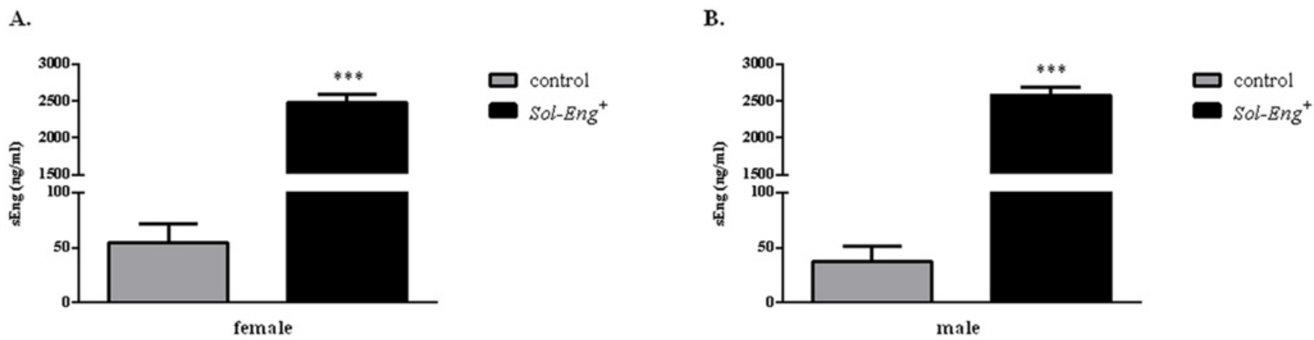

Fig 1. Concentrations of human soluble endoglin in plasma of *Sol-Eng*$^+$ and control mice. Human soluble endoglin concentrations in plasma from female (control n = 53, *Sol-Eng*$^+$ n = 22) (A) and male (control n = 31, *Sol-Eng*$^+$ n = 19) (B) mice. Data are shown as mean ± S.E.M. Mann-Whitney test, ***p≤0.001.

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respectively) (Fig. 5A). The effect of L-NAME on PHE-induced constriction was also similar in all experimental groups (females from *Sol-Eng*$^+$ Δ = 92.17 and control mice Δ = 102.7; males from *Sol-Eng*$^+$ Δ = 97.8 and control mice Δ = 124.5) (Fig. 5B).

No change in the expression of markers for endothelial dysfunction in aorta in *Sol-Eng*$^+$ mice

Western blot analysis was performed in order to evaluate the changes in the aortic expression of endoglin, eNOS, ICAM-1 and VCAM-1 between *Sol-Eng*$^+$ and control groups. Western blot analysis revealed no significant differences in endoglin and eNOS expression in aorta between *Sol-Eng*$^+$ and control mice (Fig. 6A-D). In addition, no changes in the expression of cell adhesion molecules ICAM-1 and VCAM-1 were detected in *Sol-Eng*$^+$ mice in comparison with control animals (Fig. 6E-H).

Alterations in vascular contractility in female *Sol-Eng*$^+$ mice

In order to evaluate a possible impact of high soluble endoglin levels on aortic function, vascular contractility was evaluated in *Sol-Eng*$^+$ and control mice. Receptor-independent vascular contraction induced by KCl (30 mM) was similar in aorta taken from each experimental group (control female, *Sol-Eng*$^+$ female, control male, *Sol-Eng*$^+$ male; Fig. 7A). Also, prostaglandin F2α (PGF2α)-induced vasoconstriction was not different between *Sol-Eng*$^+$ and control female mice (212.3±21.06 *vs*. 254.2±18.22, respectively) as well as between *Sol-Eng*$^+$ and control male mice (254.6±17.87 *vs*. 254.2±26.57, respectively) (Fig. 7B). In contrast, the vasoconstrictor response to phenylephrine (PHE) was significantly reduced in female *Sol-Eng*$^+$ mice when

Table 1. Systolic arterial pressure in conscious male and female *Sol-Eng*$^+$ mice and controls, assessed by tail cuff.

	male		female	
	control	*Sol-Eng*$^+$	control	*Sol-Eng*$^+$
mean	110.5	121.7	108.5	122.2
S.E.M.	1.9	2.3	2.2	4.2
n	10	12	10	11
p		≤0.05		≤0.05

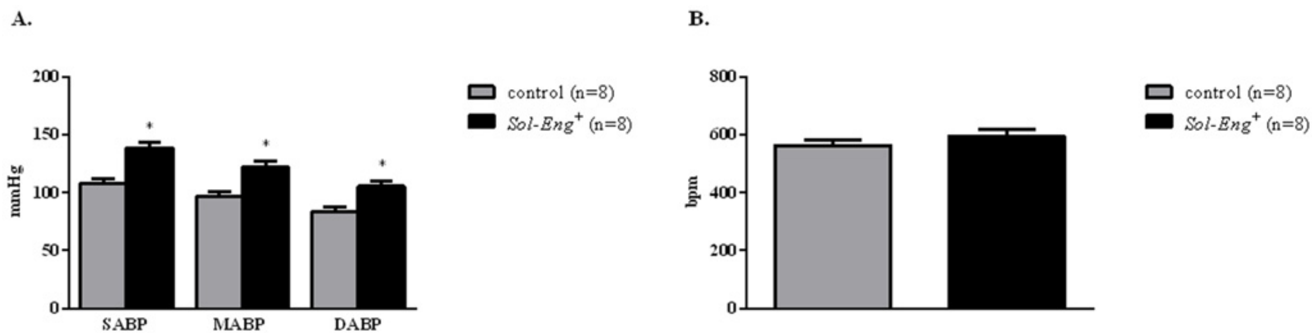

Fig 2. Blood pressure (A) and heart rate (B) in *Sol-Eng*$^{+}$ and control male mice assessed by telemetry. SABP: Systolic arterial blood pressure; DABP: Diastolic arterial blood pressure; MABP: mean arterial blood pressure. Data are shown as mean ± S.E.M. ANOVA and unpaired t-test with respect to control mice, *$p \leq 0.01$.

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compared to control mice (75.53±15.39 *vs*. 139.3±7.83, respectively), while in male *Sol-Eng*$^{+}$ this response was not altered (123.2±18.98 *vs*. 132.1±15.23, respectively) (Fig. 7C). Dose-response curves for PGF2α and PHE are shown in Fig. 7D and 7E, respectively.

DISCUSSION

A soluble form of endoglin (sEng) was demonstrated to be elevated in the sera of preeclamptic women, correlating with disease severity [3]. In addition sEng appears to contribute to the pathogenesis of pre-eclampsia by impairing binding of TGF-β1 to its receptors and downstream signaling, including effects on activation of eNOS and vasodilation, suggesting that sEng deregulate TGF-β signaling in the vasculature and may play a role in vascular dysfunction [3]. Blann et al. originally detected increased levels of sEng in hypercholesterolemic patients [6]. These authors proposed that high levels of sEng might be related to development of endothelial dysfunction in patients with hypercholesterolemia because sEng levels correlated with cholesterol levels [6]. More recently, a positive correlation between sEng plasma levels and basal glycaemia, glycated hemoglobin, endothelial dysfunction and retinopathy in patients with type II diabetes, hypertension and patients with high cardiovascular risk was observed [9]. Cui et al. showed that sEng levels and hs-CRP levels are higher in patients with plaque rupture and unstable plaques when compared with patients with stable atherosclerotic plaques [21].

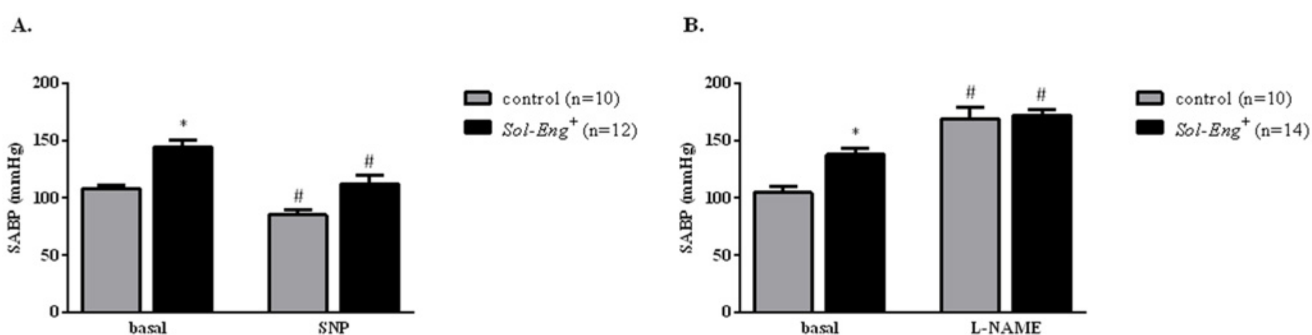

Fig 3. Pressure responses to agonists or antagonists of the NO-cGMP-system in *Sol-Eng*$^{+}$ and control mice. Maximal hypotensive effect of sodium nitroprusside (SNP; 2 mg/Kg b.w.) in *Sol-Eng*$^{+}$ and control mice (A). Maximal hypertensive effect of L-NAME (50 mg/Kg b.w.) in *Sol-Eng*$^{+}$ and control mice (B). Effects were measured by telemetry. SABP: Systolic blood arterial pressure. Data are shown as mean ± S.E.M. ANOVA and unpaired t-test with respect to control, *$p \leq 0.05$; ANOVA and paired t-test with respect to basal conditions, $^{\#}p \leq 0.05$.

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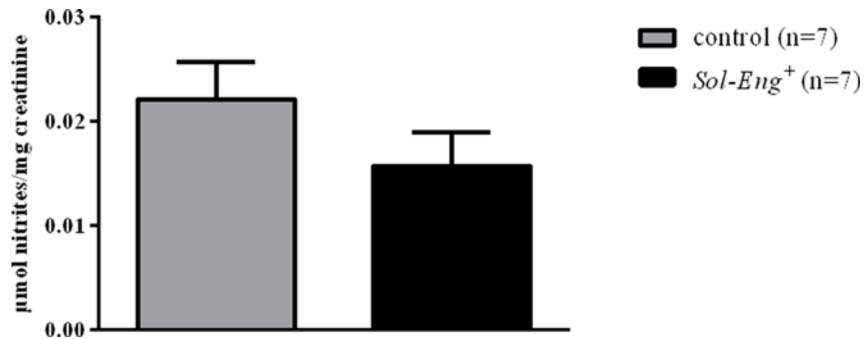

Fig 4. Urinary excretion of nitrites in *Sol-Eng*$^{+}$ and control mice. Urinary excretion of nitrites was measured in urine from *Sol-Eng*$^{+}$ and control mice collected in metabolic cages, and corrected by creatinine concentration. Data are shown as mean ± S.E.M. Unpaired t-test.

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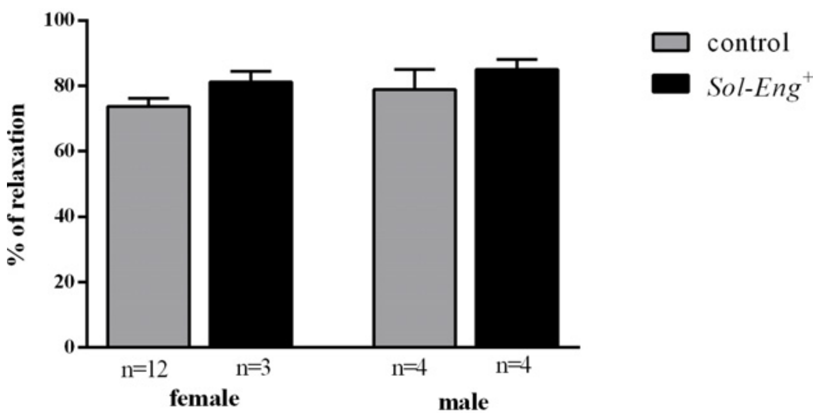
A.



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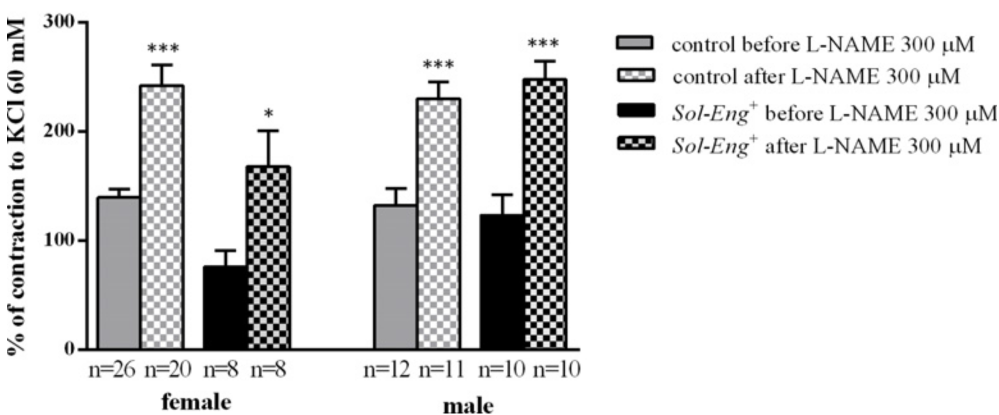

Fig 5. Endothelium-dependent responses in *Sol-Eng*$^{+}$ and control mice. Acetylcholine-induced relaxation in PHE or PGF2α (1 μM) pre-constricted vessels (A). Effect of L-NAME on the PHE (1 μM)-induced contraction (B). Data are shown as mean ± S.E.M. Mann-Whitney test, unpaired t-test, *$p \leq 0.05$, ***$p \leq 0.001$.

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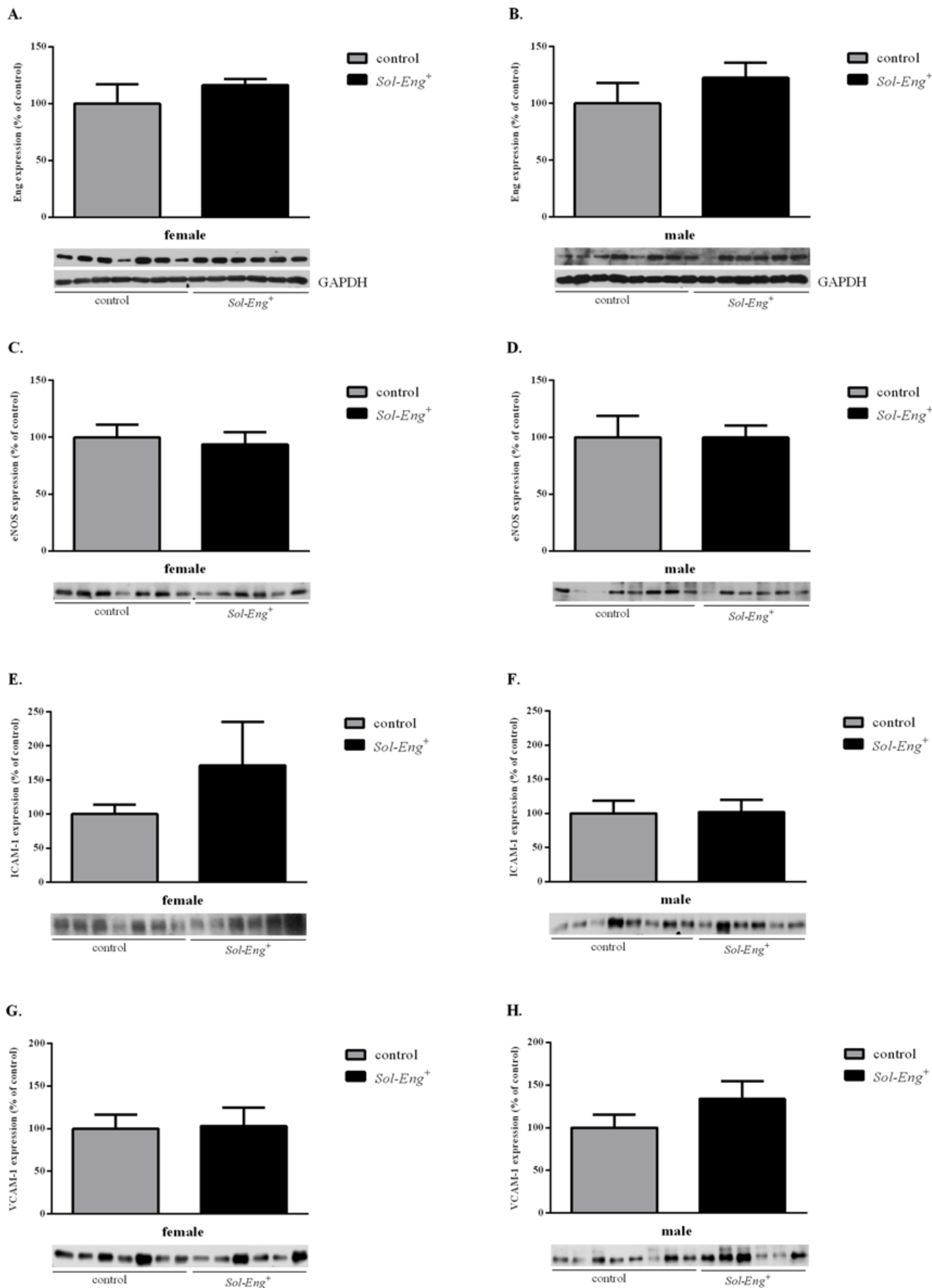

Fig 6. Markers of endothelial dysfunction in aorta. Expression of endoglin (A, B), eNOS (C, D), ICAM-1 (E, F) and VCAM-1 (G, H) in total protein extracts from mice aorta. Equal loading of samples was confirmed by immunodetection of GADPH (A, B). Top: densitometric analysis (control = 100%). Bottom: representative immunoblots. Data are shown as mean ± S.E.M. Mann-Whitney test, unpaired t-test.

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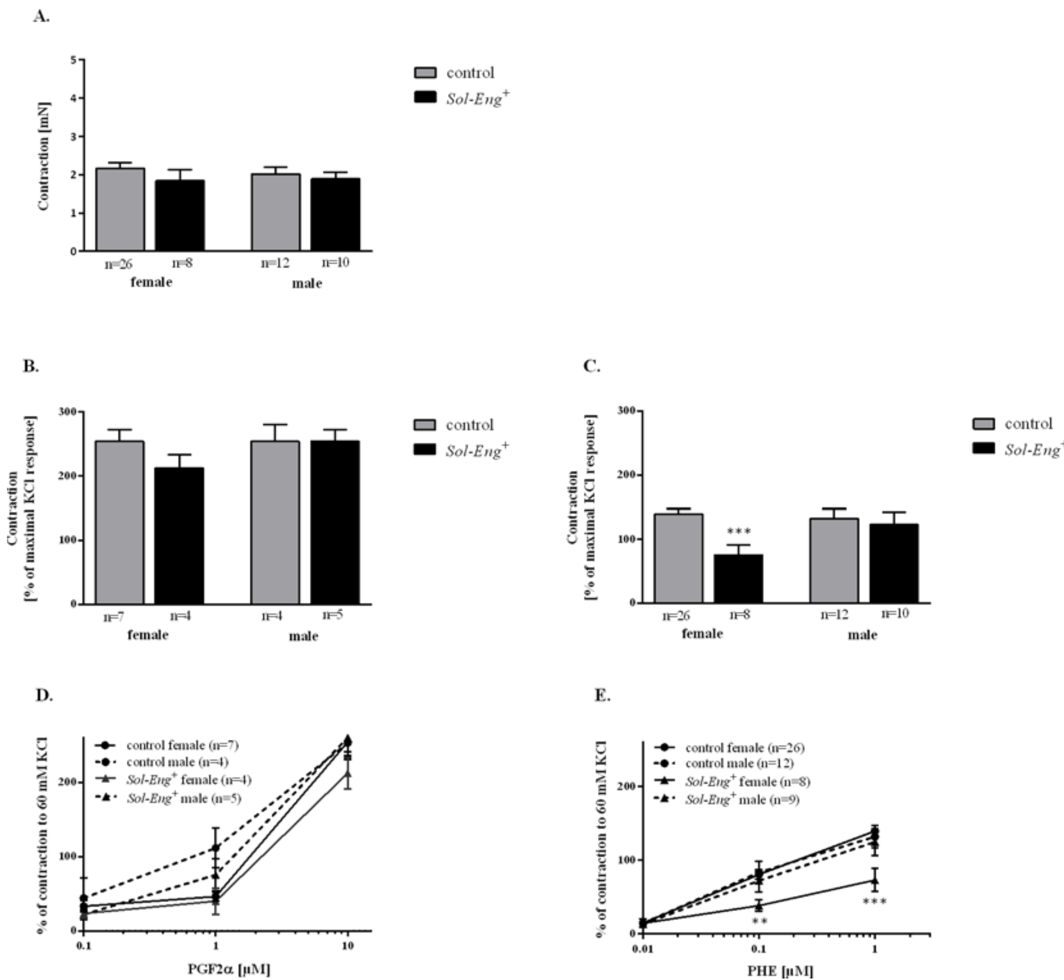

Fig 7. Impaired vascular contractility in female *Sol-Eng*$^+$ mice as compared to control mice. Maximal contraction to KCl (30 mM) (A). Maximal contraction to PGF2α (10μM) (B) and to PHE (1 μM) (C) in *Sol-Eng*$^+$ and control mice. Comparison of dose-response to PGF2α (D) and PHE (E) in *Sol-Eng*$^+$ as compared to control mice. Data are shown as mean ± S.E.M. Unpaired t-test, $^{**}p \leq 0.01$, $^{***}p \leq 0.001$.

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Changes of sEng levels were associated with hypercholesterolemia and statin treatment in experimental atherosclerosis in mice [7,22]. Soluble endoglin was also demonstrated to increase vascular permeability [3], which is also hallmark of endothelial disturbance. All these studies, suggest that sEng might be considered as an interesting biomarker associated with hypercholesterolemia/endothelial dysfunction and atherogenesis [11].

On the other hand, a few papers showed that soluble endoglin is not only a biomarker because high levels of sEng might affect vascular endothelium function. Indeed, administration of adenoviral sEng in non-pregnant mice resulted in an increased expression of P-selectin, soluble E-selectin and VCAM-1 levels, increased number of rolling leukocytes in mesenteric venules and impaired endothelial dependent vascular autoregulation, which was related to neutralization of TGF-β signaling [12]. sEng has antiangiogenic properties, and its administration to mice induced an increase in arterial pressure [5]. As mentioned above it has been suggested that soluble endoglin interacts with TGF-β1, leading to the inhibition of binding of this protein to TGF-β receptor complex. These interactions subsequently could result in sEng-induced inhibition of TGF-β1-mediated eNOS activation in endothelial cells [1,3]. In addition, binding of soluble endoglin to BMP9 may affect the Smad1/5 signaling pathway, and, in turn, may alter

endothelial function [23]. Moreover, specific binding of a mouse soluble endoglin construct (mEng^{ECD}-mFc 27–581) to human BMP9 and BMP10 was demonstrated recently [24]. This study suggests that the interaction domains between endoglin and BMP9 are highly conserved between human and mouse species [24]. Therefore, it is expected that a similar binding between human endoglin and mouse BMP9 may also occur in our transgenic mice overexpressing human soluble endoglin.

In the light of these data, we hypothesized that high levels of soluble endoglin might induce endothelial dysfunction in systemic conduit vessels, such as aorta, in transgenic mice that express high levels of human soluble endoglin (*Sol-Eng*$^{+}$) [5].

Sol-Eng^{+} mice have high plasma concentrations of human soluble endoglin within the range of 2,000–3,000 ng/mL that are far more elevated when compared to hypercholesterolemic mice with advanced atherosclerosis, where plasma concentrations of mouse soluble endoglin amounted to 2,000–3,000 pg/mL [22], which is almost 1000x less than in mice used in this study. The transgenic littermates that do not develop high levels of human soluble endoglin were used as controls in this study. It was demonstrated that *Sol-Eng*$^{+}$ mice develop a mild hypertension and proteinuria when compared to controls [5]. However, there are no data showing that high levels of soluble endoglin can induce endothelial dysfunction in aorta of these mice.

Endothelial dysfunction is characterized by the altered vascular relaxation, due to the impaired nitric oxide (NO) bioavailability that can be the consequence of either a reduced production by endothelial nitric oxide synthase (eNOS) or an increased removal by reactive oxygen species [13,25]. In addition, NO-deficiency is associated with overexpression of proinflammatory cell adhesion molecules like VCAM-1 and ICAM-1 in endothelial cells [26]. Moreover, decreased expression of tissue endoglin resulted in a reduced eNOS expression leading to an impaired endothelium-dependent vascular function [27].

In this study, we have observed that conscious mice overexpressing human soluble endoglin show a normal hypotensive response to acetylcholine and nitroprusside, thus demonstrating a normal-NO-mediated vascular relaxation. It should be noted that the effect of acetylcholine is very short and in many cases the hypotensive effect was artifacted by the removal of the mice from their cages to inject the drug and the consequent effects on arterial pressure, thus making difficult to correctly assess the hypotensive effect of ACh. Furthermore, although administration of L-NAME induced a lower increase of arterial pressure in *Sol-Eng*$^{+}$ than in control mice, this effect can be explained by the fact that *Sol-Eng*$^{+}$ mice already have a high pressure under basal conditions, and the reflex control of arterial pressure in conscious animals prevented a further increase. In addition, the endothelium-dependent vasodilation induced by acetylcholine in PHE or PGF2α pre-contracted aorta showed no differences between *Sol-Eng*$^{+}$ and control female and male mice. Indeed, the effect of L-NAME on PHE-induced constriction was also similar in all experimental groups, which means that high levels of sEng do not modify NO production by eNOS. In addition, Western blot analysis showed no differences in the expressions of eNOS, endoglin, VCAM-1 and ICAM-1 in aorta of both male and female *Sol-Eng*$^{+}$ and control mice. Thus, we might propose that the presence of elevated human soluble endoglin levels in plasma does not modulate the expression of membrane endoglin, cell adhesion molecules or eNOS on aortic endothelium in these mice fed with chow diet. In addition, urinary nitrite excretion, a measurement of whole body NO production, was similar in *Sol-Eng*$^{+}$ and control mice. These data clearly demonstrate that high levels of soluble endoglin alone cannot induce alterations of endothelial function in mice at least in basal normocholesterolemic conditions.

Despite the lack of changes in the expression of potential markers of endothelial dysfunction we found an impaired vascular response to PHE-induced contraction but not to PGF2α- or

KCl-induced contraction in female *Sol-Eng*$^+$ mice, as compared to control female mice. By contrast the response in male *Sol-Eng*$^+$ was not altered. According to the above-mentioned results we can rule out that endothelium is involved in this impaired vascular contractility of female *Sol-Eng*$^+$ mice. We might speculate that specific impairment of α1 adrenergic receptors-dependent signaling in smooth muscle cells present only in female *Sol-Eng*$^+$ mice, in addition to the differential gender-specific hormones, might be involved. This phenomenon however requires a further investigation.

It is of interest to mention that transgenic mice with high levels of soluble endoglin on any atherosclerotic background (apoE-deficient, LDLR-deficient) are not available and thus we cannot evaluate whether high levels of soluble endoglin might contribute to endothelial dysfunction in atherosclerosis where hypercholesterolemia and inflammation are also present.

In conclusion, we demonstrate that high concentration of soluble human endoglin in plasma alone is not able to induce endothelial dysfunction in aorta of *Sol-Eng*$^+$ mice which, however, does not rule out a possibility that soluble endoglin might contribute to alteration of endothelial function in combination with hypercholesterolemia and/or inflammation.

Author Contributions

Conceived and designed the experiments: IN JLN SC PN. Performed the experiments: IN AS BO KJ JR. Analyzed the data: IN AS BO KJ JR PF MV. Wrote the paper: IN AS BO JLN CB SC PN.

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High levels of soluble endoglin induce pro-inflammatory and oxidative stress phenotype associated with preserved NO-dependent vasodilatation in aorta from mice fed high fat diet

Katerina Jezkova[1&], Jana Rathouska[1&], Ivana Nemeckova[1], Petra Fikrova[1], Eva Dolezelova[1], Michala Varejckova[1], Barbora Vitverova[1], Kristyna Tysonova[1], Agnieszka Serwadczak[4] Elzbieta Buczek[4], Carmelo Bernabeu[2], Jose M. Lopez-Novoa[3], Stefan Chlopicki[4,5], Petr Nachtigal[1*]

[1] Department of Biological and Medical Sciences, Faculty of Pharmacy in Hradec Kralove, Charles University in Prague, Heyrovskeho 1203, Hradec Kralove, 500 05, Czech Republic

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

[3] Renal and Cardiovascular Physiopathology Unit, Department of Physiology and Pharmacology, University of Salamanca, 37007, Salamanca, Spain

[4] Jagiellonian Centre for Experimental Therapeutics (JCET), Bobrzynskiego 14, 30-348, Krakow, Poland

[5] Department of Experimental Pharmacology, Chair of Pharmacology, Medical College Jagiellonian University, Grzegorzecka 16, 31-531, Krakow, Poland

[&]these authors contributed equally to the manuscript

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

Phone/fax numbers: +420495067305/ +420495067170

Running title: Soluble endoglin and endothelial dysfunction

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Conflict of Interest: none declared.

ABSTRACT

Aims: A soluble form of endoglin (sEng) was proposed to participate on induction of endothelial dysfunction in small blood vessels. Here, we tested the hypothesis that high levels of sEng combined with high fat diet induce endothelial dysfunction in atherosclerosis prone aorta.

Methods and Results: Six-month-old female and male transgenic mice overexpressing human sEng (*Sol-Eng*$^{+}$) with low (*Sol-Eng*$^{+}$ *low*) or high (*Sol-Eng*$^{+}$ *high*) levels of plasma sEng were fed a high fat rodent diet containing 1.25% of cholesterol and 40% of fat for 3 months. Plasma cholesterol and mouse soluble endoglin levels did not differ between *Sol-Eng*$^{+}$ *high* and *Sol-Eng*$^{+}$ *low* mice. The expression of pro-inflammatory (P-selectin, ICAM-1, pNFkB, COX-2), and oxidative stress-related markers (HO-1, NOX-1, NOX-2) in aortas of *Sol-Eng*$^{+}$ *high* female mice was significantly higher than in *Sol-Eng*$^{+}$ *low* female mice. Endothelium-dependent vasodilatation induced by acetylcholine was best preserved in *Sol-Eng*$^{+}$ *high* female mice compared to *Sol-Eng*$^{+}$ *low* female mice.

Conclusion: These results suggest that high concentrations of soluble endoglin in plasma in combination with high fat diet simultaneously induce the activation of pro-inflammatory, pro-oxidative as well as vasoprotective mechanisms in mice aorta and their balance determines whether the final endothelial phenotype is adaptive or maladaptive.

Keywords:

Soluble endoglin; endothelial dysfunction; vascular contractility; mice; inflammation

INTRODUCTION

Endoglin (Eng) is a transmembrane glycoprotein also known as CD105 or TGF-β receptor III that acts as a co-receptor for members of the transforming growth factor-β (TGF-β) superfamily, including TGF-βs, bone morphogenetic proteins (BMPs) and activins. It plays a key role in various physiological and pathological processes, such as angiogenesis, hereditary hemorrhagic telangiectasia, cardiovascular development [1], atherosclerosis [2], coronary artery disease [3], and preeclampsia [4].

A soluble form of endoglin (sEng) is generated by the cleavage of the whole endoglin extracellular domain by membrane-type metalloproteinase-14 (MMP-14) at position 586, which results in release of endoglin extracellular domain to the extracellular fluid and plasma [5]. Increased levels of circulating sEng were found in various cardiovascular system-related pathological conditions like preeclampsia [4], hypertension, diabetes mellitus [6], atherosclerosis [7, 8] and hypercholesterolemia [9]. We also proposed that high levels of sEng may be considered as a biomarker related to the endothelial dysfunction [2]. Moreover, several authors have suggested the participation of sEng in the mechanisms of endothelial dysfunction. In fact, administration of sEng resulted in an inhibition of eNOS-dependent vasodilatation in isolated rat renal microvessels and mesenteric vessels [4]. In addition, an alteration of endothelium in mesenteric venules, characterized by increased expression of cell adhesion molecules and impaired vasodilatation, was observed after treatment of mice with adenovirus expressing sEng [10].

Recently, a transgenic mouse model expressing human soluble endoglin (*Sol-Eng*$^{+}$) has been generated. The *Sol-Eng*$^{+}$ mice have high levels of soluble endoglin in blood, exhibit a preeclampsia-like phenotype, including hypertension, small pup size, proteinuria and renal damage and is a potentially useful model to study the development of endothelial dysfunction [11]. However, a recent work in our laboratory failed to demonstrate that the elevated plasma

concentrations of sEng in these mice were not associated with endothelial dysfunction in aorta, neither at functional nor at morphological level [12].

Certain pathologies like hypertension, type II diabetes mellitus or atherosclerosis, are closely related to endothelial damage and/or functional alteration of endothelium. Indeed, endothelial dysfunction is accompanied with impaired nitric oxide (NO)-dependent vasodilatation, which is characterized by a reduced endothelial nitric oxide synthase (eNOS) activity and/or enhanced inactivation of NO by increased oxidative stress [13-15]. In addition, increased levels of reactive oxygen species (ROS) contribute to endothelial dysfunction and vascular remodeling, which can be compensated by an increased expression of superoxide dismutase (SOD) to maintain the redox homeostasis as the first antioxidant defense system [16]. It should be noted that low expression of Eng is associated with decreased NO-dependent vasodilatation, and lower expression of eNOS [17-19]. Moreover, increased expression of cell adhesion molecules was shown to represent a hallmark of endothelial dysfunction [13].

As it is well recognized that endothelial dysfunction can be induced by atherogenic diet and/or hypercholesterolemia itself [13], we tested the hypothesis that high plasma concentrations of sEng in *Sol-Eng*$^{+}$ mice combined with high fat diet might contribute to the development of endothelial dysfunction.

MATERIALS AND METHODS

Animals

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 [11]. Six-month-old female (n=5) and male (n=6) mice showing high plasma concentrations of sEng (*Sol-Eng*$^{+}$ *high*) were fed a high fat rodent diet containing 1.25% of cholesterol and 40% of fat (Research Diets, Inc., NJ, USA) for the following three months. Age matched female (n=7) and male (n=7) littermates with low levels of plasma sEng (below detection limit of ELISA) (*Sol-Eng*$^{+}$ *low*), were used as control mice. Both mice groups were derived from the same transgenic mouse line and have identical genotype. However, some animals (approximately 30%) did not develop high levels of circulating soluble endoglin leading to the existence of these two groups that differ from each other only in the levels of soluble endoglin (high or low). The animals were housed under a 12-h light cycle with constant temperature and humidity and had free access to tap water. No weight differences were detected between both experimental groups.

Animal studies met the accepted criteria for human care and experimental use of laboratory animals. All experiments were carried out in accordance with the directive of the EU (86/609/EEC) and all protocols were approved by the Ethical Committee for the protection of animals against cruelty at Faculty of Pharmacy, Charles University in Prague, and the Bioethics Committee of the University of Salamanca. All surgery procedures were carried out under ketamine/xylazine anesthesia, and all efforts were made to minimize the suffering of the animals.

Human and mouse soluble endoglin concentrations in plasma

Blood was extracted from a cut in the tail tip and concentrations of human soluble endoglin in plasma were determined by means of Human Endoglin/CD105 Quantikine ELISA Kit (R&D Systems, MN, USA). Concentrations of mouse soluble endoglin in plasma were determined by means of Mouse Endoglin/CD105 Quantikine ELISA Kit (R&D Systems, MN, USA) according to the manufacturer's instructions. For the specific nature of the transgenic model, the methodology of human sEng evaluation in plasma had to be adjusted in our studies (300-fold dilution of samples before ELISA measurement compared to typical 4-fold dilution in standard human studies).

Biochemical analysis

Total cholesterol levels and triglycerides were measured enzymatically by conventional enzymatic diagnostic kits (Lachema, Czech Republic) and spectrophotometric analysis (cholesterol at 510 nm, triglycerides at 540 nm, ULTROSPECT III, Pharmacia LKB Biotechnology, Sweden).

Vasomotor function analysis in isolated mouse aorta

Thoracic parts of aortas from anesthetized (mixture of ketamine 100mg/Kg and xylazine 16mg/Kg; i.p.) male (5 animals for *Sol-Eng*$^+$ *high* group and 5 animals for *Sol-Eng*$^+$ *low* group) and female (5 animals for *Sol-Eng*$^+$ *high* group and 5 animals for *Sol-Eng*$^+$ *low* group) mice were quickly removed and carefully dissected free from the surrounding tissue. Isolated aortas were placed in Krebs-Hanseleit solution, cleaned of the connective and fat tissue and cut into 4 rings (each 3 mm long). The rings were placed in organ chambers of the wire myograph (620M, Danish Myo Technology, Denmark) and mounted between 2 pins attached to an isometric force transducer with continuous recording of tension (PowerLab, LabChart, ADI Instruments, Australia) and gassed with 95% O_2 and 5% CO_2. After mounting

of rings, 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 experiment was initiated by obtaining maximum contraction in response to KCl (30-60 mM). After KCl washing, the aortic rings were pre-contracted with increasing concentrations of prostaglandin F2 alpha (PGF2α, 0.1-10 μM) to obtain approximately 80% of KCl induced contraction. The endothelium-dependent vasodilator response was induced by cumulative concentrations of acetylcholine (Ach, 0.01-1 μM). The endothelium-independent vasodilator response was induced by cumulative concentrations of sodium nitroprusside (SNP, 0.001-1 μM). NO-dependent vasodilator response to Ach was determined by analyzing the Ach effect after the administration of L-NAME.

Western blot analysis

Samples of whole aortas from female mice (5 female mice for *Sol-Eng*$^+$ *high* group and 7 female mice for *Sol-Eng*$^+$ *low* group, 6 male mice for *Sol-Eng*$^+$ *high* group and 7 male mice for *Sol-Eng*$^+$ *low* group) were homogenized in RIPA lysis buffer (Sigma-Aldrich, St. Louis, USA) as described previously [12]. 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 [12]. Membranes were developed using enhanced chemiluminescent reagents (Thermo Fisher Scientific Inc., IL, USA) and exposed to X-Ray films (Foma, Czech Republic). Quantification of immunoreactive bands on the exposed films was performed by image analysis software NIS (Laboratory Imaging, Prague, Czech Republic). Equal loading of proteins onto the gel was confirmed by immunodetection of GAPDH.

Statistical analysis

The statistical analysis was performed by GraphPad Prism 6.0 software (GraphPad Software, Inc., CA, USA). All data are presented as mean ± S.E.M. Direct group-group comparisons were carried out using Mann-Whitney test. P values of 0.05 or less were considered statistically significant.

RESULTS

Concentrations of human and mouse soluble endoglin and lipids in plasma from *Sol-Eng*$^{+}$ mice

Animals were fed a high fat diet for 3 months and then blood samples were obtained for subsequent studies. ELISA analysis was performed to assess both human and mouse soluble endoglin concentrations in studied mice. As shown in Fig. 1A, human soluble endoglin concentrations in plasma were substantially higher in *Sol-Eng*$^{+}$ *high* female mice (3480 ± 501 ng/mL) when compared to *Sol-Eng*$^{+}$ *low* female mice (undetectable levels). No differences in mouse soluble endoglin concentrations were observed between *Sol-Eng*$^{+}$ *high* female mice and *Sol-Eng*$^{+}$ *low* female mice (8.15 ± 0.55 vs. 7.93 ± 0.45 ng/mL, respectively) (Fig. 1B). Biochemical analysis showed no significant differences in total cholesterol (3.32 ± 0.32 vs. 3.95 ± 0.54 mmol/L, respectively) and triglyceride plasma concentrations (1.20 ± 0,07 vs. 1.10 ± 0,14 mmol/L, respectively) between *Sol-Eng*$^{+}$ *high* and *Sol-Eng*$^{+}$ *low* female mice (Fig. 1C, D). Human soluble endoglin concentrations in plasma were substantially higher in *Sol-Eng*$^{+}$ *high* male than in *Sol-Eng*$^{+}$ *low* male mice (2579 ± 115 vs. 38 ± 14) (Fig. 1E). No significant differences were observed in mouse soluble endoglin concentrations between *Sol-Eng*$^{+}$ *high* and *Sol-Eng*$^{+}$ *low* male mice (8.89 ± 3.71 vs. 8.73 ± 2.60) (Fig. 1F). There were no significant differences in total cholesterol (3.55 ± 0.32 vs. 4.13 ± 0.20) (Fig.

1G) and triglyceride plasma concentrations (1.61 ± 0.21 vs. 1.66 ± 0.17) (Fig. 1H) between *Sol-Eng*$^{+}$ *high* and *Sol-Eng*$^{+}$ *low* male mice.

Preserved NO-dependent vasodilatation in aortas of *Sol-Eng*$^{+}$ *high* female mice but not male mice fed high fat diet

Maximal contraction to PGF2α (10µM) was similar in both *Sol-Eng*$^{+}$ *high* and *Sol-Eng*$^{+}$ *low* female and male mice (Fig. 2A, B). Endothelium-dependent vasodilatation induced by acetylcholine in PGF2α pre-contracted aorta was impaired in *Sol-Eng*$^{+}$ *low* female mice fed high fat diet, but not impaired in *Sol-Eng*$^{+}$ *high* female mice as shown in Fig. 2C (48.7 ± 7.2 vs. 79.8 ± 4.1 at 1µM acetylcholine). Endothelium-dependent vasodilatation induced by acetylcholine in PGF2α pre-contracted aorta did not differ in male mice (25.4 ± 4.1 vs. 47.9 ± 18.5 at 1µM acetylcholine) (Fig. 2D). The inhibitory effect of L-NAME on Ach-induced vasodilatation in PGF2α-induced pre-constricted vessels was similar in *Sol-Eng*$^{+}$ *high* and *Sol-Eng*$^{+}$ *low* female and male mice (Fig. 2C, D), suggesting that Ach-induced vasodilation response was NO-dependent in both experimental groups. To evaluate endothelial-independent vasodilatation in vascular smooth muscle in female mice (with detected differences in vasodilatation), cumulative concentrations of SNP were used to induce relaxation in PGF2α pre-constricted vessels. No differences in *Sol-Eng*$^{+}$ *high* female mice in comparison to *Sol-Eng*$^{+}$ *low* female mice were observed (Fig. 2E).

Sol-Eng$^+$ high **female mice fed high fat diet have increased expression of inflammatory markers in aorta**

There were no histological/morphological changes in any aorta studied in both male and female group. The aortas did not show any alteration with respect to possible fat accumulation (fatty streaks) or any other visible changes in the vessel wall. In addition, Western blot analysis of ICAM-1, P-selectin, HO-1, endoglin, eNOS and eNOS (Ser1177) showed no significant differences between *Sol-Eng*$^+$ *high* and *Sol-Eng*$^+$ *low* male mice (data not shown), which was in line with male non-significant functional data.

Western blot analysis of aorta from female mice fed high fat diet for 3 months was performed in order to evaluate the aortic expression of inflammatory markers and markers of endothelial dysfunction. The analysis demonstrated higher expression of activated (phosphorylated) pNFκB (by 113%) (Fig. 3A), P-selectin (by 47%) (Fig. 3B), ICAM-1 (by 90%) (Fig. 3C) and COX-2 (by 111%) (Fig. 3D) in *Sol-Eng*$^+$ *high* female mice compared to *Sol-Eng*$^+$ *low* female mice. Importantly, the expression levels of these inflammatory markers did not differ in *Sol-Eng*$^+$ *high* mice compared to *Sol-Eng*$^+$ *low* mice fed chow diet in our previous study [12].

Sol-Eng$^+$ high **female mice fed high fat diet have an altered balance of the oxidative stress system in aorta**

To assess the changes of the oxidative stress system, we analyzed the expressions of NADPH oxidase 1 (NOX-1), NAPDH oxidase 2 (NOX-2) and the heme oxygenase 1 (HO-1) in aortas. Both, NOX-1 and NOX-2 are able to generate reactive oxygen species (ROS), whereas HO-1 can be induced in response to oxidative stress. Together the levels of these three proteins may provide a hint regarding the oxidative stress status of the aorta. Increased expression of NOX-1 (by 124%; Fig. 4A), NOX-2 (by 151%; Fig. 4B) and HO-1 (by 123%;

Fig. 4C) was observed in aortas of *Sol-Eng*$^{+}$ *high* mice compared to *Sol-Eng*$^{+}$ *low* female mice. To reveal the changes in antioxidant enzymes, which could potentially affect vasodilatation in aorta, the expression of SOD3 and catalase was analyzed. However, no statistically significant differences between *Sol-Eng*$^{+}$ *high* and *Sol-Eng*$^{+}$ *low* female mice were observed in the expressions of either SOD3 or catalase in aortas (Fig. 4D, E).

Sol-Eng^{+} _high_ female mice fed high fat diet have activated TGF-β/endoglin pathway in aorta

To detect possible changes in TGF-β signaling, analysis of membrane endoglin, TGF-β receptor II (TGF-β RII), Smad1/5/8, Smad2/3 and the phosphorylated forms of Smad1/5 and Smad2/3 were performed in the aorta. Increased expressions of membrane endoglin (by 68%; Fig. 5A), TGF-β RII (by 54%; Fig. 5B) and pSmad1/5 (by 82%; Fig. 5C) were observed in aortas of *Sol-Eng*$^{+}$ *high* mice compared to *Sol-Eng*$^{+}$ *low* female mice. Expression of Smad1/5/8 (Fig. 5D), pSmad2/3 (Fig. 5E) and Smad2/3 (Fig. 5F) were similar in *Sol-Eng*$^{+}$ *high* and in *Sol-Eng*$^{+}$ *low* female mice.

Protein expression of markers potentially affecting NO-dependent vasodilatation in aorta of female mice fed high fat diet

To evaluate possible changes in the expression of proteins that might be involved in the vasodilatation of aorta, we analyzed expressions of eNOS, p-eNOS (Ser1177), p-eNOS (Ser632), iNOS and VEGF (Fig. 6A-E, respectively). No significant differences in these parameters were observed between *Sol-Eng*$^{+}$ *high* and *Sol-Eng*$^{+}$ *low* female mice.

DISCUSSION

A soluble form of endoglin (sEng) in plasma was demonstrated to be cleaved from the extracellular domain of the membrane-bound endoglin, which is predominantly expressed by endothelial cells in blood vessels [5]. So far, several authors demonstrated increased plasma sEng concentration during various cardiovascular and metabolic diseases e.g. hypercholesterolemia (atherosclerosis) [9, 20], preeclampsia [21], hypertension, and type II diabetes mellitus [6]. Thus, it was proposed that soluble endoglin might be a biomarker of clinical relevance showing a progression and possibly a manifestation of the above-mentioned pathological conditions [2] that are all related to the development of endothelial dysfunction.

Indeed, some authors suggested a possible role of sEng in the induction of endothelial dysfunction. It has been shown that administration of sEng (both recombinant sEng or sEng delivered by adenovirus) resulted in an impairment of endothelial function characterized by alteration of NO-dependent vasodilatation, increased expression of cell adhesion molecules, increased permeability and leukocyte trafficking throughout the endothelium [4, 10].

In a previous study, we evaluated possible alterations in vascular functions in aorta from transgenic mice characterized by high levels of human sEng [12]. We used human sEng in order to be able to differentiate between human "transgenic" sEng and endogenous mouse sEng produced by shedding of mouse membrane endoglin. In general, there is a high level of homology between mouse and human sEng (99% sequence overlap, 69% identity) [22, 23]. In addition, the purpose to use this model in this study was to evaluate the effects of high levels of sEng in the mice aorta. The first paper demonstrating a functionality of this model was the paper by Valbuena-Diez et al. 2012 where the authors demonstrated that mice with high levels of human sEng suffer from many of the symptoms of preeclampsia, such as hypertension, renal damage and proteinuria [11].

However, we failed to detect changes in NO-dependent vasodilatation, NO production and inflammatory reaction in aorta from these mice with high levels of human sEng in plasma at the age of 6 months. Of note, these *Sol-Eng$^+$ high* mice displayed normal (low) levels of cholesterol in blood when they were fed with chow diet [12]. On the other hand, patients with atherosclerosis, hypertension or type II diabetes mellitus suffer from hypercholesterolemia or dyslipidemia. This raises the question, whether high levels of plasma sEng might have an impact on the vascular wall only in the presence of other insult such as high cholesterol induced by atherogenic diet [24] contributing to the development of endothelial dysfunction in conduit vessels in such conditions.

Therefore, in the current study, mice were fed a high fat rodent diet containing 1.25% of cholesterol and 40% of fat for 3 months. Despite the fact that a mild hypercholesterolemia was induced in both *Sol-Eng$^+$ high* and *Sol-Eng$^+$ low* mice, no differences in total cholesterol levels were detected between both groups. *Sol-Eng$^+$ high* mice had significantly higher levels of human soluble endoglin, whereas mouse soluble endoglin levels did not differ between both experimental groups. Thus, we propose that the changes observed in aorta from *Sol-Eng$^+$ high* mice and *Sol-Eng$^+$ low* mice can be exclusively attributed to high and low human plasma sEng levels.

Despite the fact that above mentioned papers showed no effect of soluble endoglin on endothelial function or induction of endothelial dysfunction, we found NO-dependent vasodilatation significantly better preserved in *Sol-Eng$^+$ high* in comparison with *Sol-Eng$^+$ low* female mice. In addition, we demonstrated that this preserved vasodilatation involved NO synthesis, since the administration of L-NAME, the most frequently used inhibitor of NO synthases [25], fully abolished the Ach-induced vasodilatation supporting the notion that the preserved Ach-induced vasodilation in *Sol-Eng$^+$ high* female mice was dependent on NO. On the other hand, no effect of high levels of sEng on either vascular contractility or protein

expression was found in male mice. There is currently no explanation for this phenomenon. Indeed, *Sol-Eng^{+} high* female mice have higher levels of soluble endoglin when compared to *Sol-Eng^{+} high* male mice. One might speculate that this fact could be critical for different response in aorta in female and male mice. Sex hormones could be also involved but there are no data studying soluble endoglin effects and sex hormones interplay. In general, sEng effects and sex differences were beyond the scope of this paper.

We aimed to elucidate a molecular background for preserved vascular contractility in *Sol-Eng^{+} high* female mice by means of Western blot analysis.

Surprisingly, we showed that *Sol-Eng^{+} high* female mice have higher expression of activated NFκB, P-selectin, ICAM-1 and COX-2 in aorta suggesting a pro-inflammatory phenotype induction in the vessel wall of *Sol-Eng^{+} high* mice, compatible with the notion that increased expressions of NFκB, P-selectin, ICAM-1 represent hallmarks of endothelial dysfunction [26].

In addition, NOX-1 and NOX-2 expressions in aortas were also higher in *Sol-Eng^{+} high* than in *Sol-Eng^{+} low* mice. Since NOXs represent enzymes producing primarily ROS, and NOX-1 and NOX-2 are present in endothelial cells [27] and in vascular smooth muscle cells [28-30], we propose that high levels of human plasma sEng in mice fed high fat diet induce oxidative stress in the aortic wall.

Taken together, mice with preserved endothelial function show pro-inflammatory oxidative stress-related changes in aorta. Thus in the next step, we focused on the elucidation of this phenomenon. We analyzed potential mechanisms that could be involved in the preservation of NO-dependent vasodilatation in *Sol-Eng^{+} high* mice exposed to high fat diet.

It was demonstrated that VEGF modulates vascular tone via Akt-dependent phosphorylation of eNOS Ser1177 (p-eNOS) [31]. Moreover, other authors proposed that

extracellular superoxide dismutase SOD3 in cooperation with catalase improves endothelial-dependent vasodilatation in various conditions by protection of the NO-mediated signaling [32-34]. Therefore, we tested the hypothesis that VEGF and SOD3 may contribute to the preserved NO-dependent vasodilatation in *Sol-Eng*$^{+}$ *high* mice. In order to prove this interpretation, we analyzed the expressions of VEGF, eNOS, p-eNOS (Ser1177), SOD3 and catalase. There were no significant differences between *Sol-Eng*$^{+}$ *high* and *Sol-Eng*$^{+}$ *low* group detected in this study. Thus, we excluded the possibility that expression changes in these markers could explain the preserved NO-dependent vasodilatation in *Sol-Eng*$^{+}$ *high* mice.

On the other hand, we found that expression of HO-1 at the protein level was increased in aorta from *Sol-Eng*$^{+}$ *high* mice fed high fat diet (Fig. 3). HO-1 is a highly inducible vascular protective enzyme activated by various stimuli, including oxidative stress (e.g. LDL oxidation) and inflammation (e.g. TNF-α) [35]. The catalytic activity of HO-1 on the heme group results in the release of carbon monoxide (CO), which, in turn, activates soluble guanylyl cyclase (sGC) similarly as NO. Thus, endothelium-derived CO diffuses to subjacent smooth muscle cells, where activation of sGC results in elevated intracellular cGMP levels leading to smooth muscle relaxation [36]. In addition, several papers demonstrated an interplay between CO and NO. CO activates sGC when NO levels are low [37] and CO was suggested to have a permissive role in NO production [38]. Moreover Wu and Wang, proposed that CO-mediated NO release could have a physiological impact on the vessel wall function [39]. Thus, we suggest that the up-regulation of HO-1 might represent a compensatory mechanism (via CO release) to counteract the increased oxidative stress in aorta from *Sol-Eng*$^{+}$ *high* mice and could participate in the maintenance of NO-dependent vasodilatory function of aorta in these mice. However, this hypothesis needs to be substantiated in further studies.

In the present study, we also found that the expression of membrane-bound endoglin, a protein predominantly expressed by endothelial cells in mouse aorta [40], is increased in aorta from *Sol-Eng*$^{+}$ *high* mice. Interestingly, membrane endoglin was shown to be related to a proper function of eNOS and NO-dependent vasodilatation [17, 41], whereas its reduced expression resulted in an impaired endothelium-dependent vasodilatation [17]. Moreover, endoglin expression was demonstrated to be up-regulated after arterial injury, suggesting that endoglin is a part of a repair/protective mechanism against vascular damage [42]. Thus, we propose that mouse endoglin might be up-regulated as a compensatory response to counteract pro-inflammatory and oxidative stress stimuli in aortas of *Sol-Eng*$^{+}$ *high* mice. We speculate that increased expression of endoglin might be, at least partially, responsible for the preserved vasodilatation in aortas of *Sol-Eng*$^{+}$ *high* mice, despite the presence of inflammation and oxidative stress. In addition, endoglin expression was also accompanied by increased expression of TGF-β RII and phosphorylated Smad1/5. Regardless some controversial effects of TGF-β1 in atherosclerosis and inflammation, TGF-β1 and TGF-β RII were demonstrated to exert anti-inflammatory effects in various cardiovascular conditions [43, 44]. Furthermore, the increased levels of endoglin are compatible with the augmented phosphorylation levels of Smad1/5 as a downstream target of the endoglin/ALK1 pathway in endothelial cells [1]. Although it has been shown that endoglin-dependent eNOS overexpression is mediated by Smad2-dependent signaling pathway and that Smad2 mediates TGF-β effects on eNOS expression [41, 45], we did not find any significant differences in Smad2/3 phosphorylation between our two experimental groups. This finding is in agreement with the equal expression levels of eNOS in both experimental groups.

Limitations of the transgenic animal model used in this study are related to very high levels of soluble in *Sol-Eng*$^{+}$ *high* mice. To the best of our knowledge, there is not any murine model of transgenic mice overexpressing mouse soluble endoglin. Among the reasons for

using this transgenic model overexpressing human soluble endoglin, are: i) the availability of this animal model that has been well characterized [11]; (ii) the functional effects of human soluble endoglin in this model have been well documented [11, 12]. In addition, as discussed above, murine and human endoglin are functionally equivalent. Since, it is just an animal model that does not fully reproduce human diseases where sEng is upregulated, such as preeclampsia, the levels of sEng cannot be extrapolated to humans. On the other hand, despite these limitations, we propose that it is a useful animal model to study "proof of concept" that high sEng levels are not only a biomarker of cardiovascular pathology, but also even a possible inducer of endothelial changes, most likely in both microvasculature and conducting atherosclerosis-prone vessels. Moreover, it is of interest to mention that we did not measure blood pressure in this study, however previous studies have shown that *Sol-Eng high*$^{+}$ mice have slightly higher blood pressure than *Sol-eng*$^{+}$ *low* mice [12]. Moreover, these authors showed that the differences in blood pressure between *Sol-Eng*$^{+}$ *high* and *Sol-Eng*$^{+}$ *low* did not affect vascular endothelium with respect to the function and proteins expression. So basically, we propose in this study that high fat diet should represent a stronger and a critical factor affecting endothelium and the vessel wall in the presence of high sEng levels.

In conclusion, the results of this study show that combination of risk factors (hypercholesterolemia/high fat diet) and high levels of soluble endoglin affect functional and morphological properties in aorta. Female mice with high plasma concentration of human sEng fed high fat diet display endothelial dysfunction-like phenotype characterized by inflammation and oxidative stress in aorta, which was, quite surprisingly, accompanied by a preserved NO-dependent vasodilatation (preserved endothelial function) most likely due to increased HO-1 and membrane endoglin expression. These results suggest that sEng effects in the vessel wall are not necessarily a cause of endothelial dysfunction at least in the early phase of endothelial dysfunction development. Alternatively, these results suggest that after

three months of high fat diet, high concentration of soluble endoglin in plasma induced simultaneously activation of pro-inflammatory, oxidative stress and vasoprotective mechanisms in the vessel wall and the balance among these biological processes determines the outcome. These double sword effects of sEng in atherosclerosis-prone aorta will be further evaluated in long-term study, which perhaps finally elucidate the role of sEng with respect to the induction of endothelial dysfunction.

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Fig. 1. Plasma concentrations of human (A, E) and mouse (B, F) soluble endoglin, total cholesterol (C, G) and triglycerides (D, H) in female (A, B, C, D) and male (E, F, G, H) mice fed high fat diet for three months. Human soluble endoglin levels in *Sol-Eng*$^{+}$ *low* female mice were below detection limit. Data are shown as mean ± S.E.M. Mann-Whitney test, ***p≤0.001.

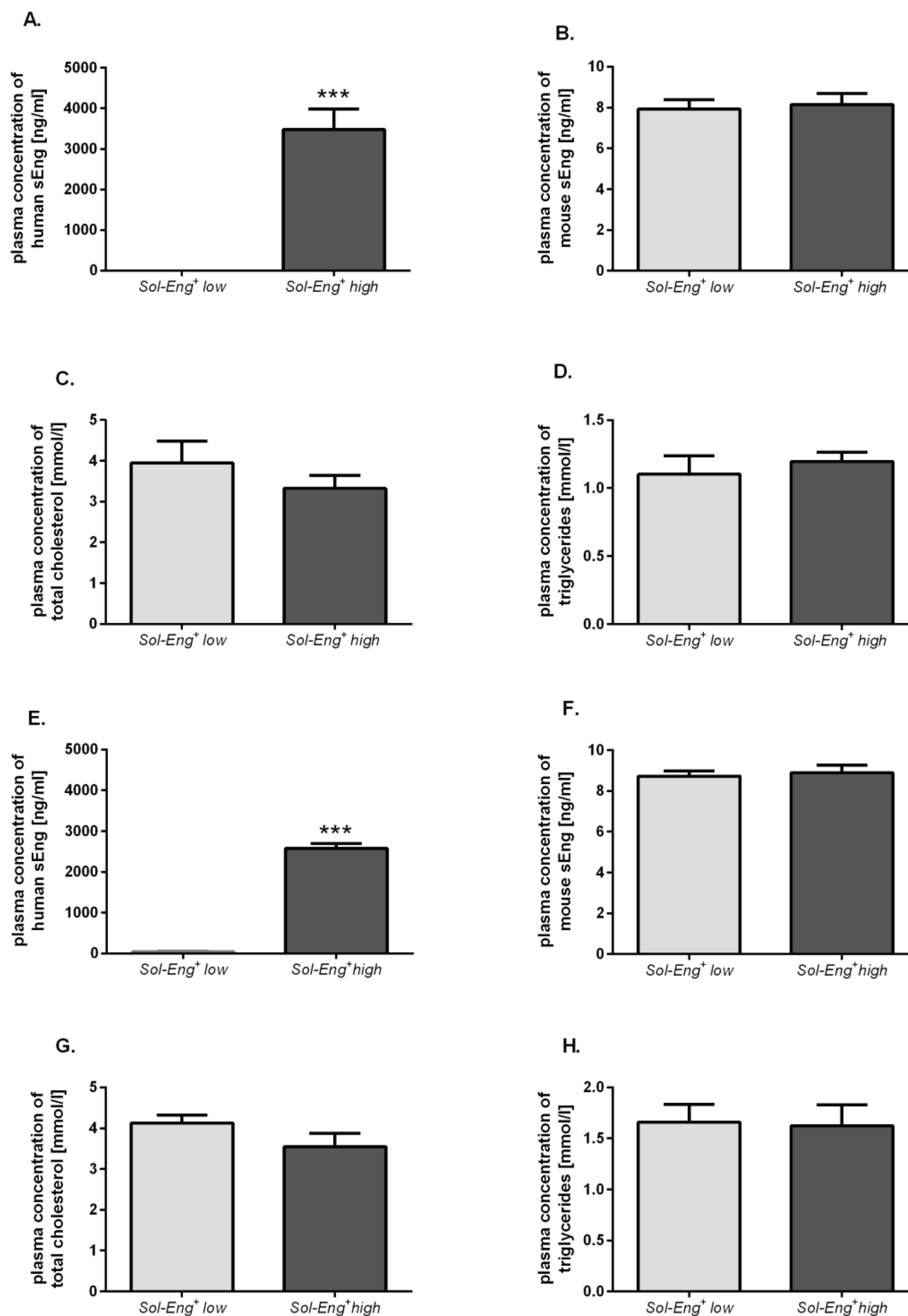
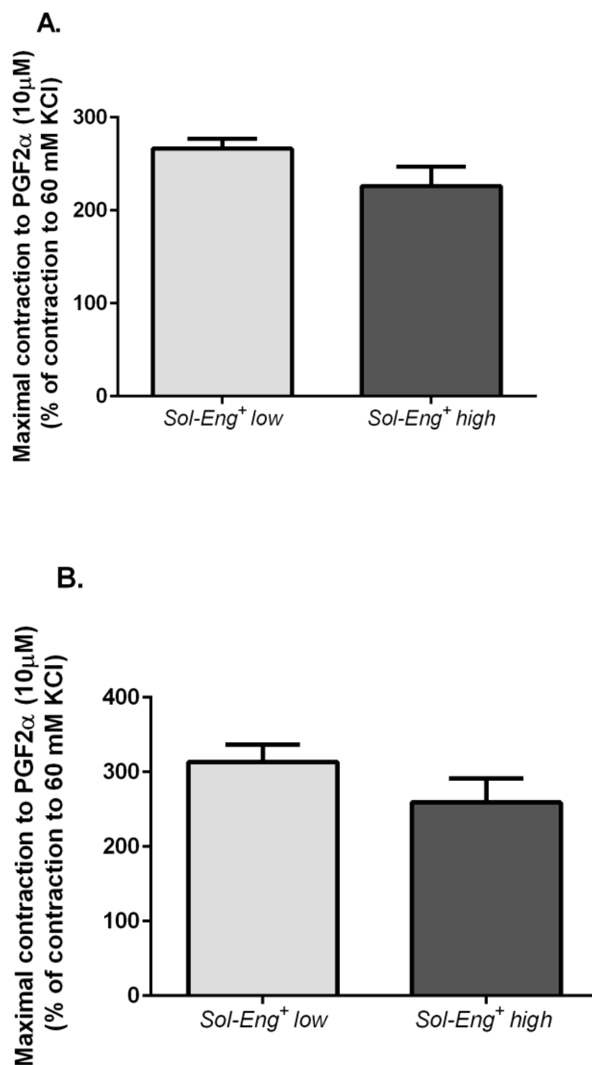
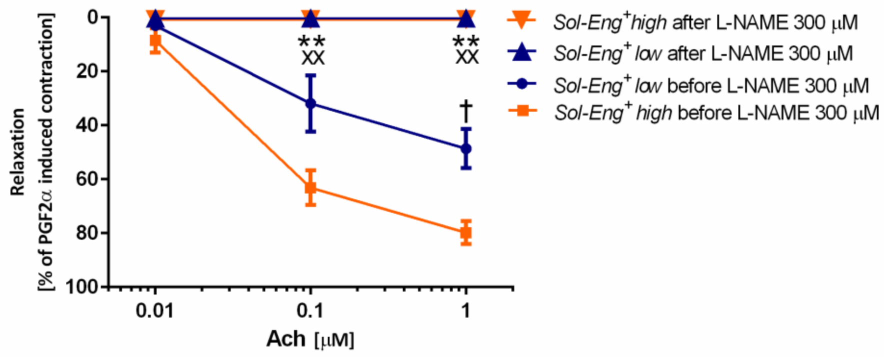


Fig. 2. Endothelium-dependent response in *Sol-Eng*$^{+}$ *high* and *Sol-Eng*$^{+}$ *low* female and male mice fed high fat diet for 3 months. Maximal contraction to PGF2α (10μM) (female – A, male - B). Acetylcholine-induced relaxation in PGF2α pre-constricted vessels (female – C, male - D). Effect of L-NAME on the endothelium-dependent relaxation (female – C, male - D). SNP-induced relaxation in PGF2α pre-constricted female vessels (E). Data are shown as mean ± S.E.M. Mann-Whitney test. **p≤0.01 (*Sol-Eng*$^{+}$ *low* before *versus* after L-NAME); ^{xx}p≤0.01 (*Sol-Eng*$^{+}$ *high* before *versus* after L-NAME); †p≤0.05 (*Sol-Eng*$^{+}$ *low versus Sol-Eng*$^{+}$ *high* before L-NAME).




C.
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20
40
60
80
100
Relaxation
[% of PGF2α induced contraction]
0.01
0.1
1
Ach [μM]
**
XX
**
XX
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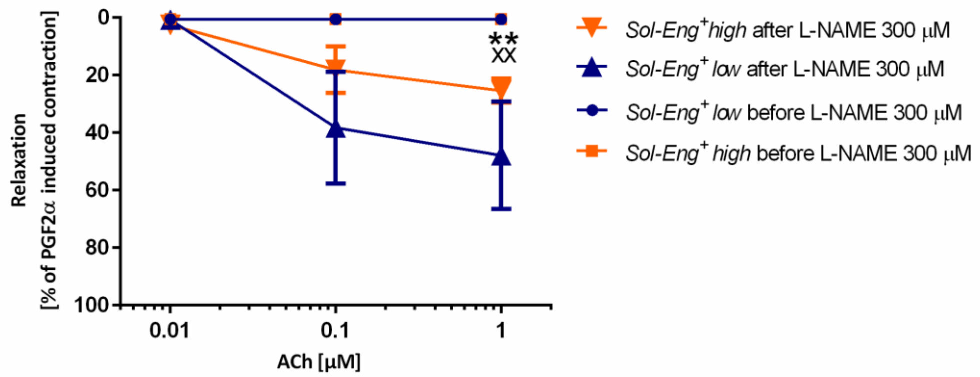
Sol-Eng+ high after L-NAME 300 μM
Sol-Eng+ low after L-NAME 300 μM
Sol-Eng+ low before L-NAME 300 μM
Sol-Eng+ high before L-NAME 300 μM


D.
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Relaxation
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ACh [μM]
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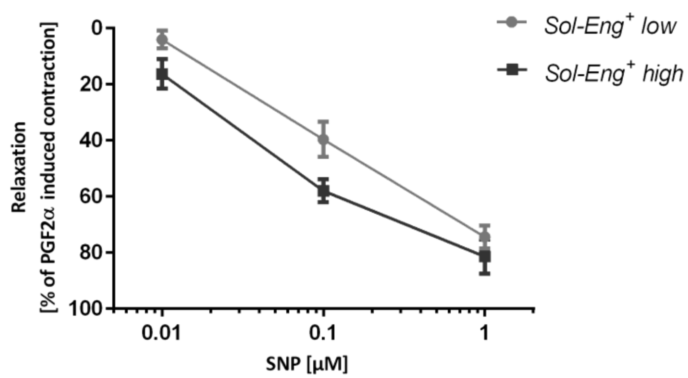
Sol-Eng+ high after L-NAME 300 μM
Sol-Eng+ low after L-NAME 300 μM
Sol-Eng+ low before L-NAME 300 μM
Sol-Eng+ high before L-NAME 300 μM


E.
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Relaxation
[% of PGF2α induced contraction]
0.01
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1
SNP [μM]
Sol-Eng+ low
Sol-Eng+ high

Fig. 3. Expression of inflammatory markers in *Sol-Eng*$^+$ *high* and *Sol-Eng*$^+$ *low* female mice fed high fat diet for 3 months. Expression of pNFκB (A), P-selectin (B), ICAM-1 (C) and COX-2 (D) in total protein extracts from mice aortas. Top: densitometric analysis (control = 100%). Equal loading of samples was confirmed by immunodetection of GAPDH (A). Bottom: representative immunoblots. n=7 for *Sol-Eng*$^+$ *low.* n=5 *Sol-Eng*$^+$ *high.* Data are shown as mean ± S.E.M. Mann-Whitney test, **p≤0.01.

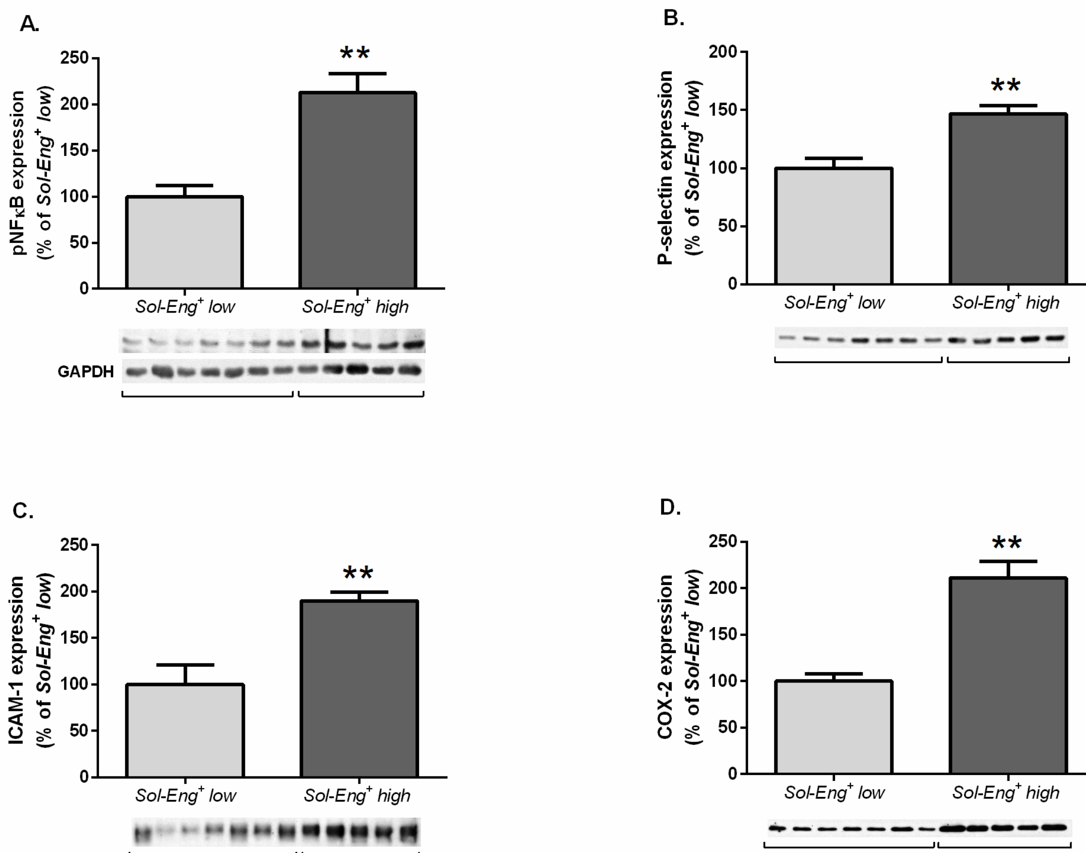


Fig. 4. Expression of some components of the oxidative stress system in *Sol-Eng*$^+$ *high* mice and *Sol-Eng*$^+$ *low* female mice fed high fat diet for 3 months. Expression of NOX-1 (A), NOX-2 (B), HO-1 (C), SOD3 (D) and catalase (E) in total protein extracts from mice aortas. Top: densitometric analysis (control = 100%). Bottom: representative immunoblots. n=7 for *Sol-Eng*$^+$ *low*. n=5 *Sol-Eng*$^+$ *high***.** Data are shown as mean ± S.E.M. Mann-Whitney test, **p≤0.01.

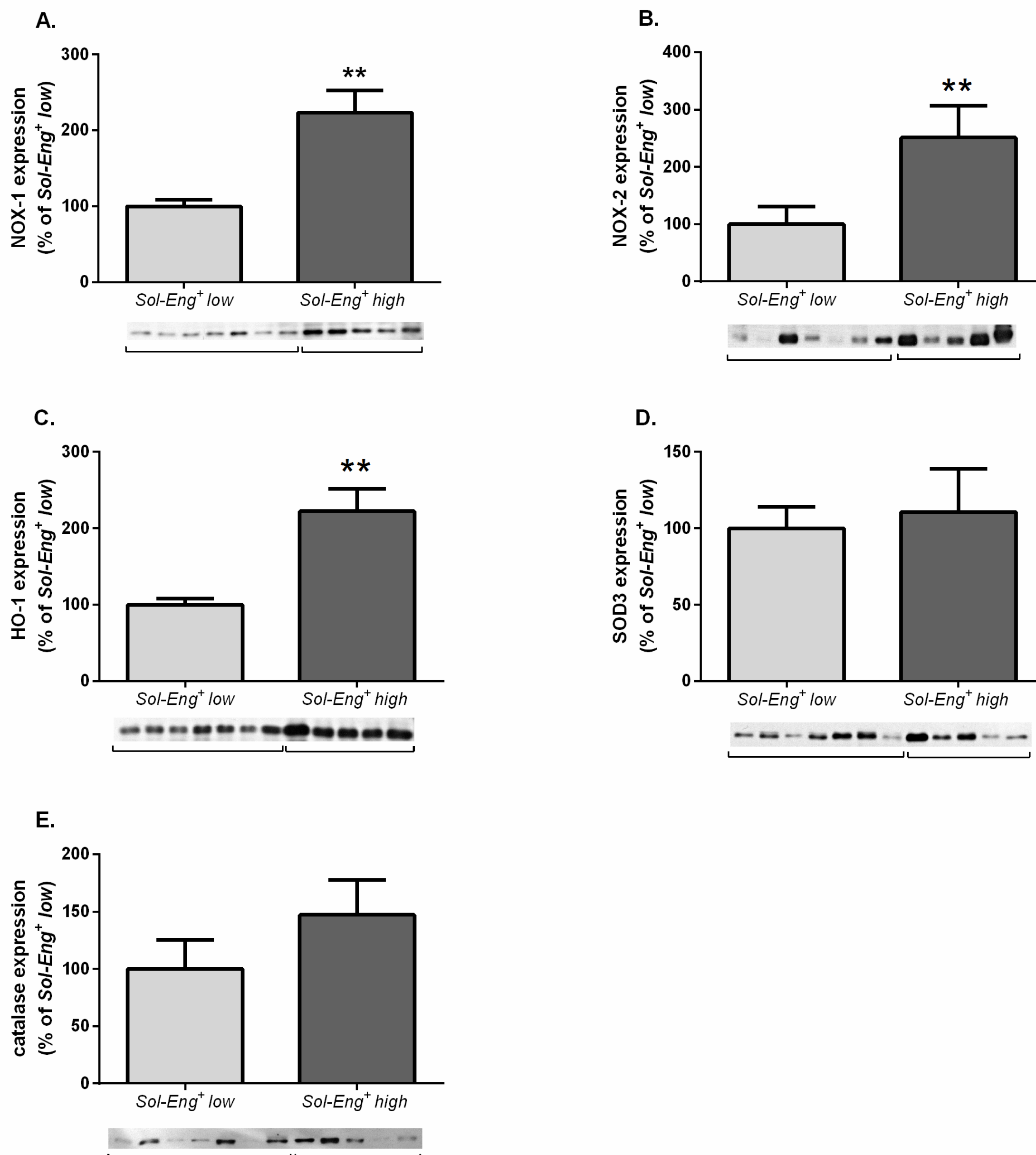


Fig. 5. TGF-β signaling components in *Sol-Eng*⁺ *high* mice and *Sol-Eng*⁺ *low* female mice fed high fat diet for 3 months. Expression of membrane endoglin (A), TGF-β RII (B), pSmad1/5 (C), Smad1/5/8 (D), pSmad2/3 (E) and Smad2/3 (F) in total protein extracts from mice aortas. Top: densitometric analysis (control = 100%). Bottom: representative immunoblots. n=7 for *Sol-Eng*⁺ *low*. n=5 *Sol-Eng*⁺ ***high*.** Data are shown as mean ± S.E.M. Mann-Whitney test, *p≤0.05.

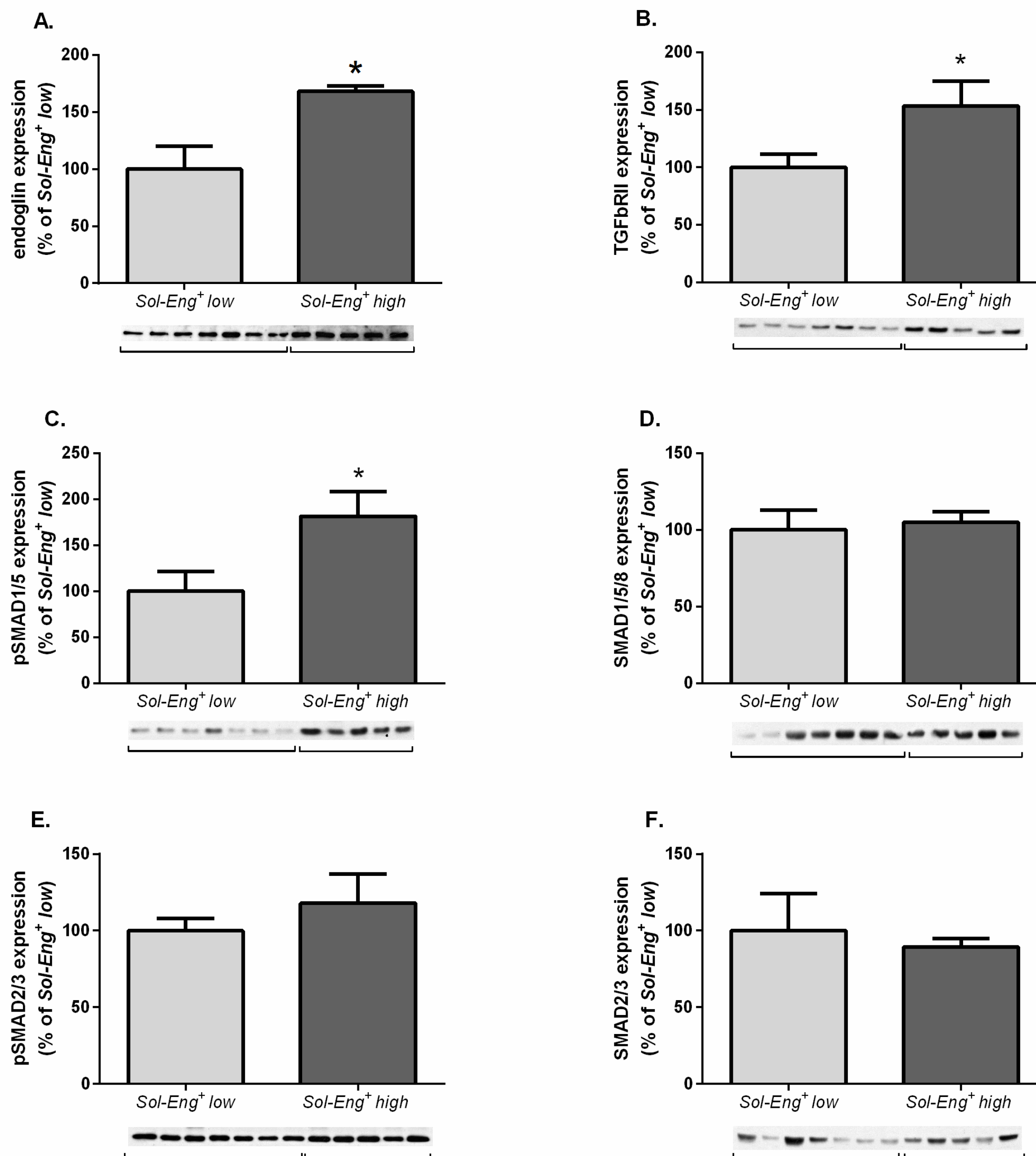
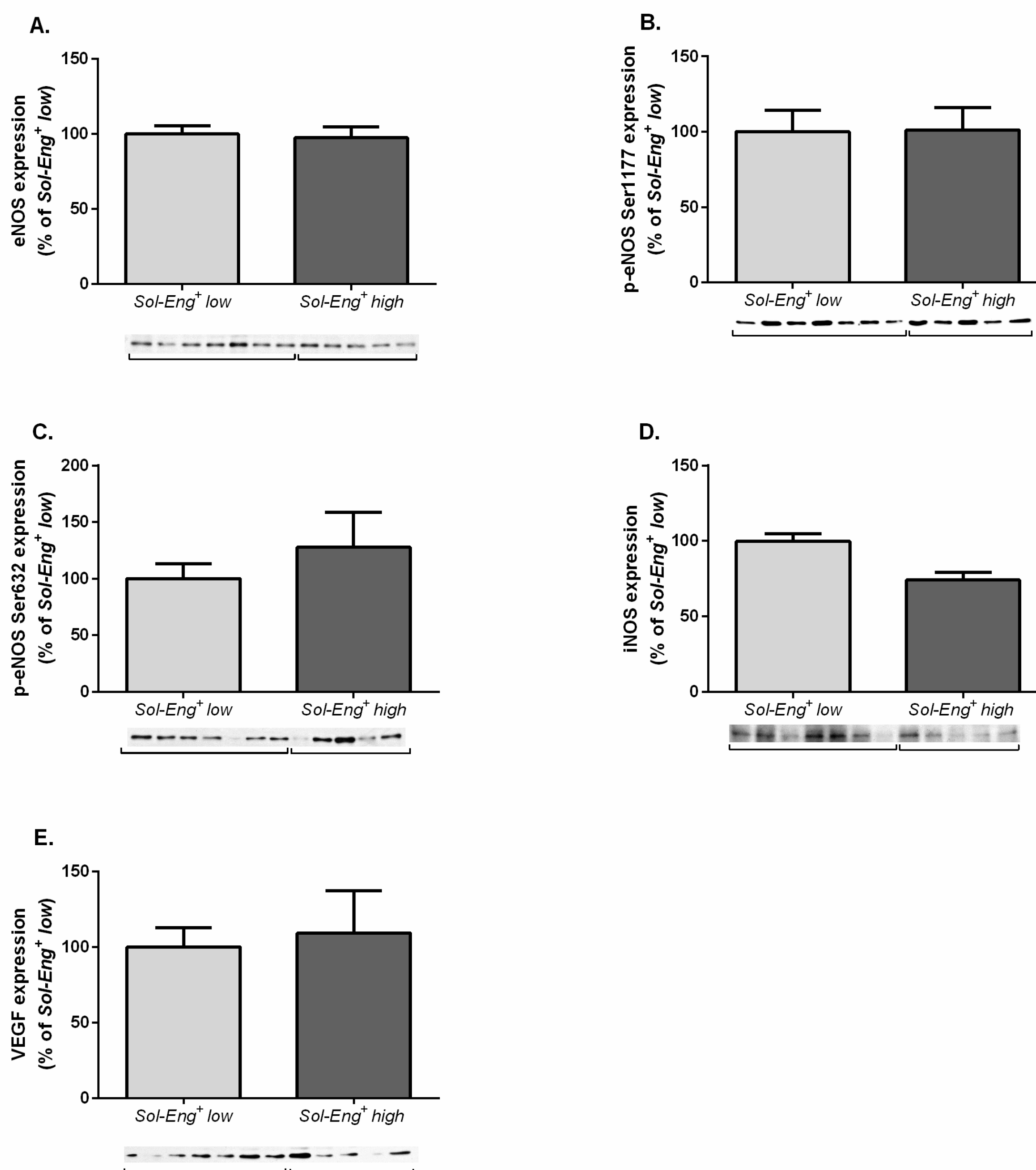
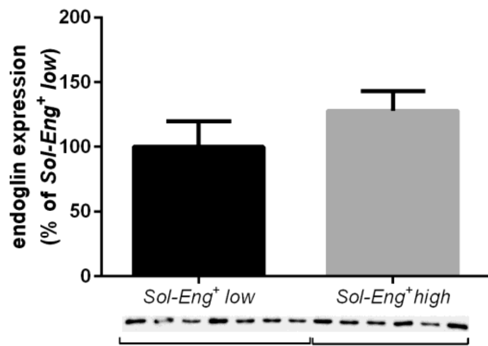
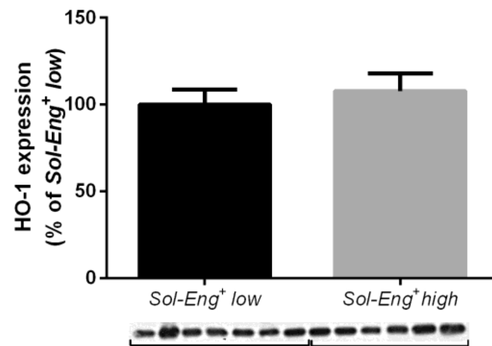


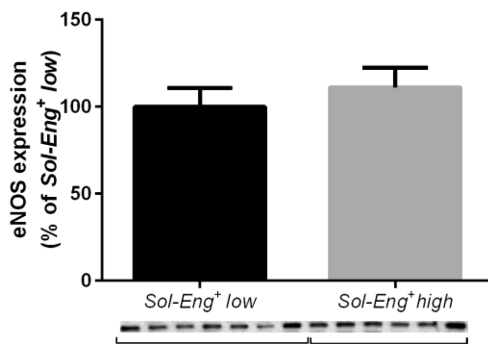
Fig. 6. Expression of various nitric oxide synthases and VEGF in aortas from *Sol-Eng*$^{+}$ *high* and *Sol-Eng*$^{+}$ *low* female mice fed high fat diet for 3 months. Expressions of eNOS (A), p-eNOS (Ser1177) (B), p-eNOS (Ser632) (C), iNOS (D) and VEGF (E) in total protein extracts from mice aortas. Top: densitometric analysis (control = 100%). Bottom: representative immunoblots. n=7 for *Sol-Eng*$^{+}$ *low*. n=5 *Sol-Eng*$^{+}$ *high.*

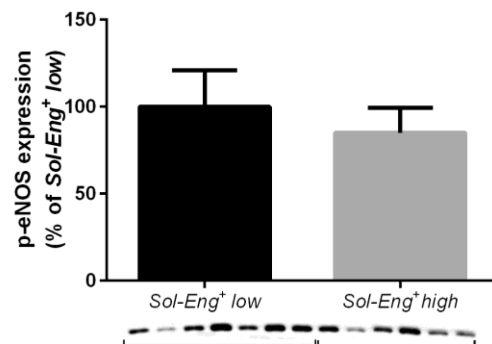


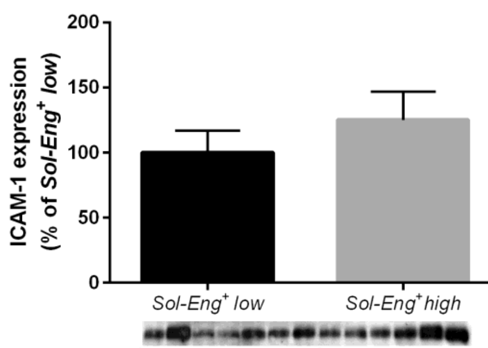
Supplementary material: MALE results

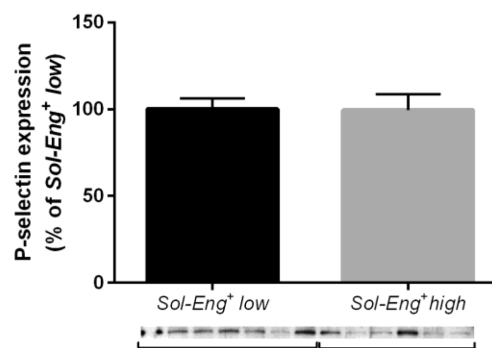


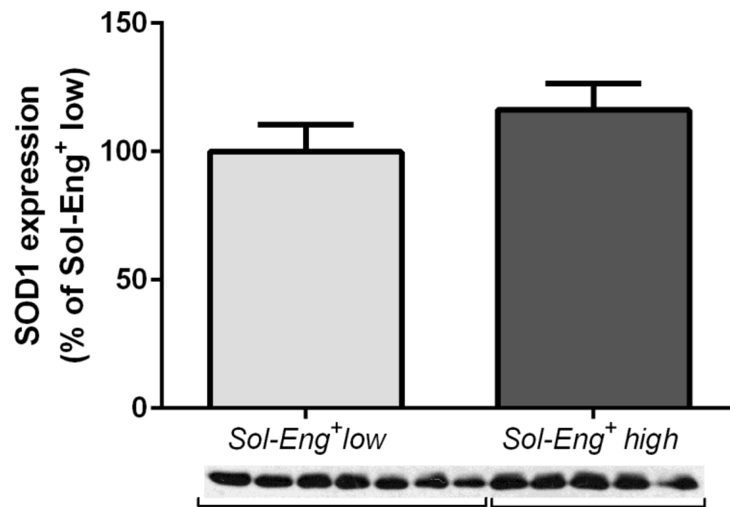











SOD1 expression
(% of Sol-Eng+ low)
150
100
50
0
Sol-Eng+low
Sol-Eng+ high

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Review article

Soluble endoglin, hypercholesterolemia and endothelial dysfunction

Jana Rathouska, Katerina Jezkova, Ivana Nemeckova, Petr Nachtigal*

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

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ABSTRACT

A soluble form of endoglin (sEng) is known to be an extracellular domain of the full-length membrane endoglin, which is elevated during various pathological conditions related to vascular endothelium.

In the current review, we tried to summarize a possible role of soluble endoglin in cardiovascular pathologies, focusing on its relation to endothelial dysfunction and cholesterol levels. We discussed sEng as a proposed biomarker of cardiovascular disease progression, cardiovascular disease treatment and endothelial dysfunction. We also addressed a potential interaction of sEng with TGF-β/eNOS or BMP-9 signaling.

We suggest soluble endoglin levels to be monitored, because they reflect the progression/treatment efficacy of cardiovascular diseases related to endothelial dysfunction and hypercholesterolemia. A possible role of soluble endoglin as an inducer of endothelial dysfunction however remains to be elucidated.

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1. Introduction

Endoglin is a homodimeric transmembrane glycoprotein, also called TGF-β receptor III or CD105. There are two forms of endoglin currently studied with respect to many physiological and pathological states. Specifically, a membrane form expressed in various tissues and a soluble form (sEng) found in plasma of healthy people as well as in patients suffering from a variety of diseases. Several review papers discussed the role of the membrane endoglin in many cardiovascular pathologies, including hereditary hemorrhagic telangiectasia [1], preeclampsia [2], atherosclerosis [3], and cancer [4]. In addition, circulating levels of the soluble endoglin have been also reviewed in the context of preeclampsia and cancer diseases [5,6]. However, a possible role of sEng with respect to endothelial function/dysfunction, hypercholesterolemia and atherogenesis has not been summarized so far. The aim of the present review was then to resume the current knowledge about the changes of sEng during hypercholesterolemia, development of endothelial dysfunction and atherogenesis, and with respect to a possible role of sEng as an inducer of endothelial alteration.

* Corresponding author.
E-mail address: nachti@faf.cuni.cz (P. Nachtigal).

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2. Generation of soluble endoglin

A soluble form of endoglin (sEng) is known to be an extracellular domain of the full-length membrane endoglin entering the systemic circulation in various conditions related to endothelial injury, activation, inflammation and senescence of endothelium [7]. After testing several matrix metalloproteinases (MMPs) in HUVECs, sEng has been proposed as an N-terminal endoglin cleavage product chipped at position 586 predominantly by membrane-type metalloproteinase-14 (MMP-14) [8]. In addition, MMP-14 was demonstrated to be the most abundantly expressed metalloproteinase in endothelial cells. The authors proposed that MMP-14 might play an important role in endoglin shedding in patients with preeclampsia or cancer [8]. Moreover, the role of MMP-14 in endoglin shedding was demonstrated after oxysterol treatment that activated LXR transcription factor and MMP-14 expression in Jar cells and placental explants. In the same study, high levels of plasma sEng have been also detected in mice overexpressing MMP-14 [9]. On the contrary, Brownfoot et al. did not confirm these results and whilst there was a modest upregulation of sENG from HUVECs there was no change in sENG secretion from primary trophoblasts. Furthermore, MMP-14 was not upregulated in HUVECs suggesting that perhaps MMP-14 may not be the primary cleavage protease [10]. In addition, no data about the role of MMP-14 in endoglin cleavage during atherogenesis and/or hypercholesterolemia are available now.

Apart from the awareness of the chipping enzyme, the real position of the membrane endoglin cleavage in various pathologies is also under investigation. An 80-kDa molecule of sEng cleaved from the surface of HUVECs at the cleavage site was shown to be at position 586, implying that the whole extracellular domain (Eng1-586) was released [8]. Thus, the Eng1-586 construct was used to generate a recombinant sEng. However, Gregory et al. purified sEng from the sera of preeclamptic women, showing a 65-kDa band, as opposed to the 80-kDa mass of recombinant Eng1-586, suggesting that the circulating sEng observed in preeclampsia is not cleaved at the position 586. Instead, the purified endoglin from sera of preeclamptic patients was identified of a lower molecular mass with C-terminal shortly after residue 406 [2]. It is of interest to mention that no similar study has been performed in the field of atherosclerosis, so there is currently no information about the structure of sEng cleaved from atherosclerosis-prone arteries (e.g. aorta). The chipping position of the tissue endoglin in various diseases is then a subject of relevant up-to-date studies, still being not fully clarified.

3. Soluble endoglin, hypercholesterolemia and endothelial dysfunction in various cardiovascular pathologies

Hypercholesterolemia is one of the most studied risk factors resulting in endothelial dysfunction and atherosclerosis. The first reference about the relation of cholesterolemia and sEng came from the work of Blann et al., demonstrating increased serum levels of sEng in patients with atherosclerosis. They found it associated with total cholesterol levels but not with other markers of endothelial damage or dysfunction e.g. E-selectin [11]. In the study of Li et al., they speculated that sEng levels increased in early stages of atherosclerosis due to the damage of endothelial cells and then decrease in later stages of atherosclerosis because of increased formation of CD105/TGF-β1 complexes [12]. sEng has been qualified as a marker increasing with high total cholesterol levels in patients with familial hypercholesterolemia [13].

In addition to hypercholesterolemia, other cardiovascular risk factors could affect soluble endoglin levels. From the point of pathophysiology of endothelial dysfunction and atherosclerosis, inflammation and oxidative stress play a crucial role. Soluble endoglin levels were increased after the treatment with inflammatory cytokine TNF-α and after the induction of oxidative stress by H_2O_2 [14]. On the other hand, it was demonstrated that a vessel-protective HO-1 inhibits sEng release from endothelial cells and placenta explants [15]. Other risk factors related to the development of endothelial dysfunction and atherosclerosis include arterial hypertension and type II diabetes mellitus. In line with this notion, serum sEng levels were proposed to be a possible indicator of hypertension and diabetes-associated vessel pathologies [16]. The study analyzed 288 patients with type II diabetes, hypertension and healthy controls showing significant correlations between endoglin and glycemia, glycated haemoglobin, systolic blood pressure, left ventricular hypertrophy and endothelial dysfunction. In addition, sEng levels were higher in patients with diabetes suffering from diabetic complications (retinopathy), and in patients with diabetes and hypertension when compared to healthy controls [16].

Coronary circulation and heart vessels were also studied with respect to soluble endoglin levels. Soluble endoglin has been proposed as an indicator of endothelial senescence, inflammation and oxidative stress in heart vessels showing that the membrane endoglin cleavage simply reflects the vascular damage (a direct proportion of damage and sEng levels), which corresponds to the adverse events in patients with coronary artery disease [14]. The elevation of sEng levels was also related to the atherosclerotic plaque morphology and correlated with unstable angina pectoris, acute myocardial infarction and post infarction heart remodeling [17].

A different point of view was presented in the study of Cruz-Gonzales et al. In the context of an acute myocardial infarction, they speculated that levels of sEng might reflect a membrane endoglin expression in the heart. They demonstrated that the decrease in sEng levels in patients with the poorest prognosis might be related to reduced expression of tissue/membrane endoglin regardless of MMPs expression, suggesting some kind of balanced ratio between the membrane and the soluble form [18]. Moreover, they also showed sEng levels to be lower in patients with acute myocardial infarction when compared with healthy subjects proposing that reduced sEng levels may reflect an impaired endothelial function. Finally, they suggested that early soluble endoglin decrease might be a novel prognostic marker of an early cardiovascular death [18].

Several papers also mentioned therapeutical interventions affecting soluble endoglin levels. Blaha et al. showed that LDL apheresis reduced levels of blood cholesterol particles followed by reduction of sEng and other biomarkers of endothelial dysfunction (hs-CRP and sCD40L) in patients with familial hypercholesterolemia. Thus, the observed drop of sEng levels was not attributed to LDL apheresis itself, but to decreased activity of endothelial cells and immune system following the removal of atherogenic elements [13]. In addition, Brownfoot et al. found that oxysterols increase sEng release from primary human tissues, however with no effect of pravastatin treatment on sEng levels [10].

In addition, it is of interest to point out that there is currently no evidence showing any correlation between changes of sEng levels in blood and the expression of its membrane form in aorta or any other specific organ. Thus, a direct link between therapeutical intervention and sEng levels still needs to be considered carefully.

In our previous studies with apoE/LDL receptor (apoE/LDLr) double knockout mice, blood soluble endoglin and cholesterol levels were increased and atherosclerotic plaques were naturally bigger after the administration of cholesterol-rich diet. Moreover, we detected a reduced expression of the membrane endoglin in aortas of these mice [19]. In addition, we revealed that atorvastatin treatment is able to reduce the levels of cholesterol and plaque size as well as the levels of soluble form of endoglin, and simultaneously increase expression of its membrane form in aorta [20].

After five-year continual studies on several mouse models of atherosclerosis [19–22], we summarized our results describing various stages of the atherosclerotic process and cholesterol levels facing the appropriate levels of sEng to reveal any possible relationship between these values. We tried to compare these parameters in various groups of mice, specifically in C57BL/6J mice on chow diet, in apoE-deficient mice on either chow diet or Western type diet containing 21% fat (11% saturated fat) and 0.15% of cholesterol, and in apoE/LDLr double knockout mice fed chow or cholesterol diet containing 1% of cholesterol. It is essential to mention that both apoE-deficient and apoE/LDLr deficient mice represent mouse models of atherosclerosis that develop spontaneous hypercholesterolemia, endothelial dysfunction and atherosclerosis [23]. These processes can be accelerated by administration of various types of cholesterol-rich diets [24]. Our data showed that sEng levels were significantly increased in apoE/LDLr deficient mice fed cholesterol diet when compared to C57BL/6J mice, apoE deficient mice and apoE/LDLr deficient mice fed chow diet (Fig. 1A). It must be stressed that apoE/LDLr deficient mice fed cholesterol diet also reached the highest cholesterol levels of all studied groups (Fig. 1B). In addition, a clear correlation between the mean group total cholesterol levels and their relevant mean group levels of sEng was found (Fig. 1C). Surprisingly, atherosclerotic plaque sizes in various groups of mice (Fig. 1D) did not correlate with either sEng or cholesterol levels. In accordance with the previous study of Blaha et al., we might speculate that hypercholesterolemia induced in blood vessels might be related to changes of

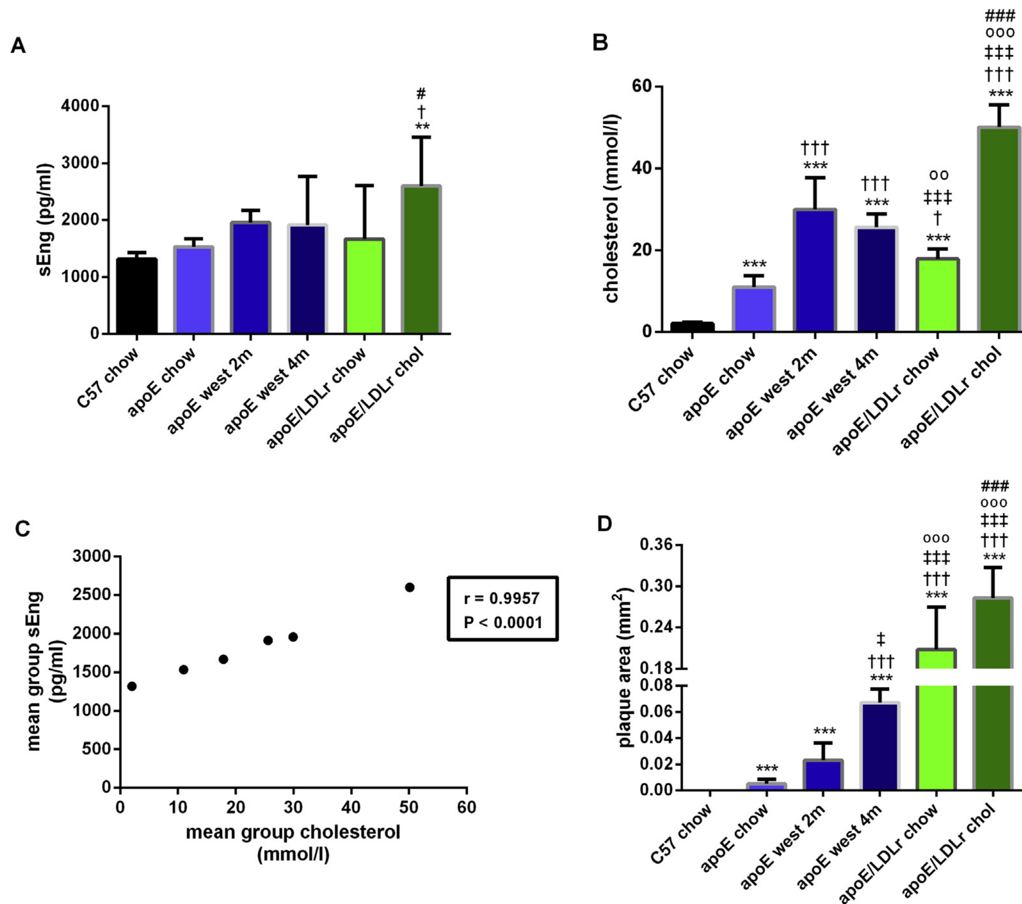

Fig. 1. Levels of soluble endoglin (A), total cholesterol levels (B) and soluble endoglin/total cholesterol correlation (C), and corresponding plaque sizes (D) in various mouse models of atherogenesis. C57 chow – C57BL/6J mouse strain fed chow diet till the age of 5 months, apoE chow – apoE deficient mice fed chow diet till the age of 4.5 months, apoE west 2 m – ten-week-old apoE deficient mice fed Western type diet for the following two months (till the age of 4.5 months), apoE west 4 m – ten-week-old apoE deficient mice fed Western type diet for the following four months (till the age of 6.5 months), apoE/LDLr chow – apoE/LDLr double knockout mice fed chow diet till the age of 4 months, apoE/LDLr chol – eight-week-old apoE/LDLr double knockout mice fed cholesterol diet for the following 2 months (till the age of 4 months). Values are means ± SEM, *$p < 0.05$, **$p < 0.01$, ***$p < 0.01$ (other symbols corresponding); * compared to C57 chow; † compared to apoE chow; ‡ compared to apoE west 2 m; O compared to apoE west 4 m; # compared to apoE/LDRr chow.

sEng levels in blood. It is also necessary to note that aorta may not be the only source of soluble endoglin released into the circulation. It is more likely that hypercholesterolemia induces the cleavage of endoglin also from other blood vessels. In line with this notion, sEng levels were not related to the plaque size, plaque composition and atherogenesis in aorta. Since we clearly demonstrated tissue endoglin expression only in endothelial cells in mice [25], we suggest that hypercholesterolemia affects sEng levels in blood by chipping endoglin in different blood vessels from vascular endothelium only.

4. Soluble endoglin as a possible inducer of endothelial dysfunction in various parts of vascular bed

Endothelial dysfunction is characterized by shifting of the physiological properties of the vessel towards a vasoconstrictor, prothrombotic and proinflammatory state [26]. The feature accompanied by increased expression of adhesion molecules, decrease in amount of several substances supporting vasodilatation and increase in vascular permeability facilitating immune cells enter the subendothelial space causing an inflammatory response to slowly accumulating molecules of cholesterol characterize the early stage of atherogenesis [27].

As mentioned above, sEng might be considered as a biomarker of endothelial damage. The question is whether sEng may be, at least partially, directly responsible for induction/development of endothelial dysfunction. Venkatesha et al. performed several experiments showing potential effects of sEng on endothelium or endothelial cells [7]. They showed that sEng is able to inhibit tube formation, suggesting its antiangiogenic effects. In addition, BALB/c mice pretreated with adenovirus expressing sEng showed increased capillary permeability in the lungs, liver and kidney. Moreover, administration of recombinant sEng to rat interfered with TGF-β1 binding to TGF-β receptor II and downstream signaling (reduced Smad2/3 activation), which resulted in an inhibition of eNOS-dependent vasodilatation in isolated rat renal microvessels and mesenteric vessels [7], suggesting possible hypertensive effects of sEng. This observation is in line with the feature of preeclampsia, a disease associated with hypertension and endothelial dysfunction, where sEng levels represent a disease severity biomarker [7,28]. In another study, Walshe et al. demonstrated alteration of endothelium in mesenteric venules after using adenovirus expression of sEng (Ad-sEng). Ad-sEng expression resulted in a neutralization of TGF-β and VEGF effects with a subsequent increase in expression of P-selectin and leukocyte rolling to endothelium, elevated levels of soluble E-selectin and soluble VCAM-1 and impaired vasodilatation [29]. On the other hand, Rossi et al. proposed sEng to inhibit leukocyte adhesion in venules as opposing the membrane endoglin action, however without any proposed mechanistic background [30]. Thus, most of the data strongly suggest that sEng might induce signs of endothelial dysfunction, however it is essential to note that none of these studies showed effects of sEng in atherosclerosis-prone arteries.

A different approach to study the role of sEng was demonstrated recently. Transgenic mice overexpressing either human sEng (sEng levels higher than 2000 ng/ml) or MMP-14 were used. MMP-14 overexpression resulted in higher levels of sEng in blood and in an increase in systolic blood pressure when compared to wild type littermates. Similarly, mice overexpressing human soluble endoglin had higher systolic blood pressure when compared to wild type littermates [9]. Despite the fact that the precise mechanism of high sEng levels inducing high blood pressure was not revealed in this study, we might speculate that this effect could be due to high levels of sEng interfering with the TGF-β/TGF-β receptor II pathway and the subsequent inhibition of eNOS-dependent vasodilatation as mentioned above. Similarly, our recent study with the same transgenic mouse strain put on a standard laboratory diet confirmed an increased systolic blood pressure in high soluble endoglin mice in comparison with their low soluble endoglin levels littermates. On the other hand, we did not prove any contribution of high sEng levels to alteration of aortic endothelial function either at protein or at functional level, suggesting possibly no contribution to endothelial dysfunction when sEng operating as a single factor [31]. In all, it seems that sEng affects various parts of vascular bed differently and one might expect different effects in aorta, muscular arteries and also in veins/venules. In addition, it is necessary to evaluate whether a combination of several factors that contribute to endothelial dysfunction, e.g. hypercholesterolemia, may have any additive effect on endothelial function/dysfunction together with high levels of sEng.

5. A proposed mechanism of soluble endoglin action in vascular endothelium

Several possible effects of sEng on vascular endothelium have been described so far. A possible role of sEng in affecting vascular permeability, cell adhesion molecules expression or vascular tone is still missing. Consequently, its responsibility for development of endothelial dysfunction, hypertension and other vascular pathologies also remains unclear.

Several papers demonstrated that sEng is able to interact with TGF-β. In TGF-β signaling, sEng role in regulating TGF-β responses was considered rather as the opposite – soluble endoglin as a naturally occurring antagonist of TGF-β. The first paper mentioning sEng-TGF-β interactions showed that increased levels of circulating sEng resulted in decreased TGF-β actions related to proatherogenic effects [12,32]. The hypothesis demonstrated sEng to lower the concentrations of the active, unbound TGF-β [33] leading in atherogenesis. On the other hand, TGF-β exerts a "double-edge sword" effect on atherosclerosis development. In the initial stages of atherogenesis, TGF-β might promote atherogenesis by stimulating collagen production and plaque growth, in the later stages, it can be anti-atherosclerotic and can even contribute to a more stable plaque phenotype (more fibrosis and less risk of rupture, less inflammation), while in the final stages of atherogenesis, again, it can contribute to plaque growth and occlusion [34–37]. Then, drug-mediated effects on TGF-β signaling may be a part of a vessel-protective therapy. Specifically, acetylsalicylic acid administration inducing TGF-β signaling pathway results in an inhibition of vascular smooth muscle cells (VSMC) proliferation and possibly atherosclerotic plaque growth [38]. Similarly, pioglitazone-mediated activation of TGF-β/Smad 2 signaling results in a pro-apoptotic effect on VSMC, suggesting also protective effects in type II diabetes associated vascular complications [39].

As mentioned above, sEng competed with TGF-β1 binding to its receptors, interfered with downstream signaling and attenuated eNOS activation in endothelial cells [7]. It is tempting to suggest that inhibition and/or blockage of eNOS activity/function could be critical for possible effects of sEng in vascular endothelium. Indeed, a decrease in eNOS function was related to increased cell adhesion molecules expression, increased vascular permeability, anti-angiogenic effects, alteration of vasodilatatory properties of arteries, development of endothelial dysfunction, arterial hypertension and atherosclerosis [40], all actions possibly attributed to sEng.

Several studies suggested the simple idea of a direct binding of TGF-β to sEng in the circulation. Thus, it was proposed that in endothelial cells, the membrane endoglin is already in complex with TGF-β receptors and therefore the added sEng competes with TGF-β for receptor binding, resulting in TGF-β binding inhibition. On the other hand, in some cell lines lacking endogenous endoglin, the presence of sEng appeared to facilitate the binding of TGF-β to its receptors [41]. Although some authors experienced a kind of a direct binding through a co-immunoprecipitation [42], the doubt is still existing. Specifically, when measuring the strength of the bond of the membrane TGF-β receptor II with TGF-β and soluble endoglin with TGF-β, the experimental data revealed that the affinity of the first couple is much higher than the affinity of sEng to TGF-β [2,43], suggesting at least "an unequal competition" between these receptors for TGF-β molecules. Moreover, it was previously described that even the membrane endoglin is able to bind only a small portion of TGF-β with a high affinity in endothelial cells [44] and simultaneously, that the binding is possible only when endoglin associates other TGF-β receptors [3]. Why should the separated sEng then directly bind TGF-β in the circulation and even fight the bond with the membrane receptors? The final doubt is bringing the experiment where even being complexed with TGF-β receptor II, the extracellular domain of endoglin failed to bind TGF-β, suggesting that maybe not the extracellular but even the intracellular domains of TGF-β receptors are those promoting the interaction with the cytokine [45].

Contrary to these findings, a recent study dealing with the ability of sEng to scavenge TGF-β in endothelial cells, the authors contradicted a direct binding of these molecules and rather tended to a possibility of a direct binding of sEng with another biologically active molecule called bone morphogenetic protein 9 (BMP-9) [2]. They demonstrated that soluble TGF-β receptor II (sTβRII) could easily scavenge TGF-β1 and block Smad 1,5,8 phosphorylation while sEng could not. On the other hand, sEng scavenged BMP-9 and blocked BMP-9 induced Smad 1,5,8 phosphorylation [2]. This is in line with a previous publication where sEng-BMP-9 binding was also favoured [45]. The question is whether Smad 1,5,8 phosphorylation blockage can have any impact on endothelial function/dysfunction, which is the information still not available in the literature. In general, sEng could not directly scavenge TGF-β1, unless a complex of sEng-sTβRII was present. In addition, since BMP-9 does not affect eNOS phosphorylation and activation, it was proposed that sEng could not act by preventing this process [2]. On the contrary, we cannot rule out a possibility that sEng was already complexed with sTβRII, thus inhibiting TGF-β1 induced eNOS activity in previous studies [7,12].

In general, the impact of the BMP-9 binding in the vessel pathology remains elusive. Only a single study demonstrated that BMP-9 (involving the ALK-1/BMP receptor II and the non-canonical p38 MAPK pathway) could induce endothelial production of the potent vasoconstrictor, endothelin-1 (ET-1) [46], which was suggested to be important for vascular stability and possibly for regulation of blood pressure (hypertension) [2].

6. Membrane endoglin, endothelial dysfunction and atherosclerosis

Membrane endoglin expression and its relation to endothelial dysfunction and atherosclerosis has been summarized in our latest

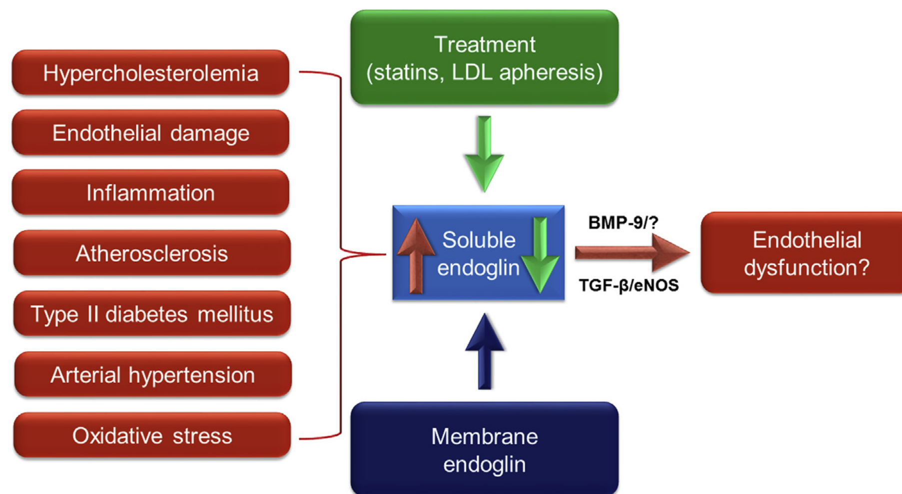

Fig. 2. Soluble endoglin and its relation to endothelial dysfunction. Hypercholesterolemia, endothelial dysfunction, inflammation, atherosclerosis, type II diabetes mellitus, arterial hypertension, oxidative stress and changes in membrane endoglin expression were related to increased levels of sEng in blood. Soluble endoglin levels might be considered as a biomarker in diseases related to endothelial dysfunction and may reflect therapeutical intervention efficacy. To date, statins and LDL apheresis were shown to reduce sEng levels. Soluble endoglin might affect TGF-β/eNOS and/or BMP-9 signaling, suggesting its role in the development of endothelial dysfunction, as demonstrated in the microcirculation so far. Adapted from [2,9,11,13,16,20,29,54].

review [3]. The atherogenic process starting from endothelial dysfunction, through the plaque growth to the plaque rupture resulting in acute coronary events has been reviewed recently [47]. Several papers showed changes of membrane endoglin expression during different stages of atherogenesis. Alteration of endoglin expression resulted in a reduced eNOS activity and impaired NO-dependent vasodilatation [48]. It was also demonstrated that endoglin can increase eNOS via a Smad 2 dependent mechanism [49]. Endoglin expression was also increased after vascular damage, suggesting that endoglin might be upregulated as a compensatory mechanism during vascular repair [50]. Moreover, endoglin expression was related to increased fibrosis and atherosclerotic plaque stability [51]. Results of these papers showed that membrane endoglin may play a protective role in the development of endothelial dysfunction and during atherogenesis as well. On the hand, several papers also demonstrated increased membrane endoglin levels correlating with disease progression. This potential controversy in membrane endoglin effects has been summarized recently [52]. It was shown that plaque vulnerability and plaque hemorrhage correlate with the presence of activated CD105 + microvessels, suggesting endoglin participating in neoangiogenesis in advanced atherosclerotic plaques, with inflammatory cells migrating to the plaque and worsening the plaque stability [53]. On the other hand, it was proposed that reduced membrane endoglin expression in the heart suffering from myocardial infarction might be related to decrease in sEng levels, suggesting sEng as possible novel prognostic marker of an early cardiovascular death [18]. More recently, membrane endoglin was shown to participate in inflammation, playing a role in leukocyte adhesion and transmigration in venules [30], the vessels, however, possessing many features far from the atherosclerosis-prone vessels. We propose that membrane endoglin expression and activity play different roles in various parts of vascular tree and its role in atherogenesis seems to be even different during the atherogenic process, which are the relations currently studied in our laboratory.

7. Conclusion

In the current review, we tried to summarize a possible role of soluble endoglin in cardiovascular pathologies, focusing on its relation to endothelial dysfunction and cholesterol levels (summarized in Fig. 2). We demonstrated:

1. sEng levels (increase) might be considered as a biomarker of various vascular pathologies related to endothelial dysfunction and hypercholesterolemia.
2. sEng levels might be monitored during the treatment of diseases where endothelial dysfunction and hypercholesterolemia play a significant role.
3. Level changes of sEng might be also related to membrane endoglin expression, however any direct evidence demonstrating changes of tissue endoglin expression in particular organ (arteries) in relation to changes of sEng levels is missing, so far.
4. sEng is able to induce signs of endothelial dysfunction, however with no direct evidence in atherosclerosis-prone arteries or during atherogenesis.
5. sEng is able to interact with TGF-β/eNOS and/or BMP-9 signaling, which might result in endothelial function impairment.

In conclusion, we suggest that sEng levels might be of interest to study with respect to the progression/treatment of cardiovascular diseases related to endothelial dysfunction and hypercholesterolemia. However, a possible role of endoglin as a potential inducer of endothelial dysfunction remains to be elucidated.

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