Atorvastatin Increases Endoglin, SMAD2, Phosphorylated SMAD2/3 and eNOS Expression in ApoE/LDLR Double Knockout Mice

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

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

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

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

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Key words: Endoglin, SMAD2, Phosphorylated SMAD2/3, eNOS, Atorvastatin, ApoE/LDLR double knockout mice

Introduction

Endoglin is a 190-kDa homodimeric transmembrane glycoprotein composed of 95-kDa disulfide-linked subunits\(^1\). The primary sequence of human endoglin is composed of an extracellular domain of 561 amino acids, a single transmembrane region, and a cytoplasmic tail\(^2\). Mutations in the gene encoding endoglin have been linked to human disease: hereditary hemorrhagic telangiectasia type 1 (HHT1), an autosomal dominant inherited vascular disorder\(^3\). Endoglin is part of the transforming growth factor-β (TGF-β) receptor cascade and is known as a type III TGF-β receptor. Endoglin forms complexes with heteromeric complexes of type I and type II serine/threonine kinase receptors (TβRI and TβRII), respectively, and has been postulated to affect TGF-β1 signaling\(^4\). Activation of TGF-β signaling results in acti-
vation and translocation of the SMAD family of proteins to the nucleus to participate in regulating gene expression\(^5\). We previously demonstrated that endoglin is expressed by the aortic vessel endothelium in normo- and hypercholesterolemic mice\(^6,7\). Moreover, endoglin expression was also detected in human and porcine atherosclerotic lesions\(^3,4,8\).

The endothelium plays a dual role in the regulation of vasomotor tone, producing and releasing both relaxing and constricting factors. The main vasorelaxing factor produced by endothelial cells (EC) is nitric oxide (NO)\(^9\). NO possesses several important biological effects, including the inhibition of cell adhesion molecule expression and thus leukocyte adhesion to the endothelium, inhibition of platelet aggregation and activation and inhibition of smooth muscle proliferation\(^10\). NO synthesis by the endothelium is maintained by endothelial nitric oxide synthase (eNOS), which is constitutively expressed but also affected by different stimuli, including hypoxia, shear stress and LDL. It has been demonstrated that alteration of eNOS expression is related to the development and progression of atherosclerosis\(^11\).

Lipid-lowering drugs offer one of the most effective therapeutic approaches used in clinical practice for the prevention and treatment of atherosclerosis. Statins, a well-known class of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors, are active in the primary and secondary prevention of coronary heart disease and are the drugs most widely used for these purposes\(^12\). Regarding the endoglin regulatory pathway, it has been demonstrated that endoglin expression by the endothelium in non-atherosclerotic vessels in mice is affected by atorvastatin treatment\(^13\) and that statins can increase eNOS expression by the endothelium\(^14\).

Recently, it has been demonstrated that endoglin expression correlates with eNOS expression and NO-dependent vasodilatation\(^15\), and that endoglin increases eNOS expression by modulating SMAD2 protein levels in endothelial cells \textit{in vitro}\(^16\); however, to the best of our knowledge, there are no available data demonstrating that endoglin co-localizes with SMAD2 and eNOS in endothelial cells \textit{in vivo}. Moreover, it is unclear whether the proteins in this pathway could be affected by statin treatment in atherosclerotic lesions.

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

**Material and Methods**

**Animals**

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

All mice were fed with different experimental diets for a further 2 months with water \textit{ad libitum} throughout the study. The control group of animals \((n=8)\) was fed a Western-type diet (atherogenic diet) containing 21% fat (11% saturated fat) and 0.15% cholesterol by weight. The same atherogenic diet and treatment period was used in atorvastatin-treated mice \((n=8)\) where atorvastatin was added to the diet at a dose of 100 mg/kg per day. The dosage of atorvastatin in this study was chosen according to both our own and others’ experiments with apoE-deficient or LDLr-deficient mice and statins, where the doses ranged from 10 mg/kg/day up to 300 mg/kg/day\(^17\). Each mouse, in both groups, lived in a separate cage and received 4 g food (in specially prepared pellets) daily. Food consumption was monitored every day. No differences in food consumption were visible, either among animals in one experimental group or between experimental groups.

At the end of the treatment period, all animals were fasted overnight and euthanized. Blood samples were collected via cardiac puncture at the time of death. Ascending aortas, were removed and then immersed in OCT (Optimal Cutting Temperature) embedding medium (Leica, Prague, Czech Republic), snap frozen in liquid nitrogen and stored at \(-80^\circ\text{C}\). Descending aortas for Western blot analysis were frozen in liquid nitrogen and stored at \(-80^\circ\text{C}\).

**Biochemistry**

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

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

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

Primary antibodies included the following: monoclonal antibody rat anti-mouse endoglin CD 105 (diluted 1:50) purchased from BD Pharmingen (CA, USA), rabbit polyclonal antibodies to phosphorylated SMAD2/3 (diluted 1:100) and eNOS (diluted 1:100), obtained from Santa Cruz Biotechnology, Inc., (CA, USA) and Rabbit polyclonal antibody directed to SMAD2 (diluted 1:30), obtained from Abcam (Cambridge, UK).

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

Western Blot Analysis

Western blot analysis was performed as described previously. Briefly, descending aortas from both groups of mice were homogenized in lysis buffer containing 10 mM Tris, 250 mM saccharose, 1 mM EDTA and protease inhibitor cocktail (Complete Mini, Roche Diagnostics GmbH, Mannheim, Germany). The homogenates were centrifuged at 2,500 rpm for 10 minutes and 10,000 rpm for 30 minutes at 4°C. The protein concentration in the supernatant was determined with the BCA Protein Assay Kit (Pierce, Rockford, IL). Samples (10 μg protein) were incubated with sample buffer at room temperature for 30 minutes and separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on 10% resp. 7.5% polyacrylamide gels. After the proteins were transferred to a nitrocellulose membrane (Bio-Rad, Hercules, CA, USA), they were blocked for 1 h with 5% nonfat dry milk in Tris-buffered saline containing 0.05% Tween 20 (TBST). The membrane was then incubated with primary antibodies (as for immunohistochemistry – see above) at the following concentrations: endoglin (90–95 kDa) and eNOS (140 kDa) at 1:500, SMAD2 (58 kDa) and phosphorylated SMAD2/3 (52 kDa) at 1:300, secondary rabbit anti-goat horseradish peroxidase-conjugated antibody at 1:5000 and horseradish peroxidase-linked donkey anti-rabbit immunoglobulin G (GE Healthcare, Prague, CZ) at 1:2500 or 1:1000. After washing with TBST buffer, the membranes were developed using SuperSignal West Femto Maximum Sensitivity Substrate (Pierce, Rockford, IL). The membranes were subsequently exposed to Hyperfilms (GE Healthcare, Prague, CZ). To quantify the bands of interest, exposed films were scanned with a ScanMaker i900 (UMAX, Prague, CZ) and quantified using QuantityOne imaging software (Bio-Rad Laboratories). Equal loading of proteins onto the gel was confirmed by immunodetection of beta-actin (anti-beta-actin antibody; Sigma, USA – diluted at 1:5000).

Statistical Analysis

All values in graphs are presented as the mean ± SEM of animals. Statistical significance in differences between groups was assessed by t-test using GraphPad Prism software (version 5.0). P values ≤ 0.05 were considered significant.

Results

Biochemistry

Biochemical analysis of blood samples from ApoE/LDLR double knockout mice showed that the administration of 100 mg/kg/day of atorvastatin resulted in a significant decrease of total cholesterol (51.9 ± 1.7
Atorvastatin treatment significantly decreased total cholesterol, LDL cholesterol, VLDL cholesterol and TAG levels when compared with control mice. Moreover, HDL cholesterol levels were increased in atorvastatin-treated groups. Values are the means ± SEM, n = 8. ***p < 0.001, *p < 0.05.

Immunohistochemical Staining of Endoglin in ApoE/LDLR Double Knockout Mice

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

Colocalization Study of Endoglin with SMAD2, Phosphorylated SMAD2/3 and eNOS in ApoE/LDLR Double Knockout Mice

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

**Western Blot Analysis**

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

**Discussion**

Endoglin (or CD105) is a homodimeric membrane glycoprotein that, in association with TGF-β receptors, binds TGF-β1 and -β3 isoforms in human endothelial cells. In addition, increased endoglin expression is observed in endothelial cells (ECs) of microvessels from pathological skin lesions and in the neovessels of tumors, suggesting a role of endoglin during endothelial cell proliferation. The role of endoglin in atherogenesis was also studied recently. Endoglin was expressed at low levels in normal porcine and human coronary arteries and overexpressed in diseased arteries, not only endothelial cells and fibroblasts but transiently in smooth muscle cells and macrophages. Moreover, its expression was detected universally in microvessels within the atheroma, suggesting its role in plaque angiogenesis.

In contrast, in this study we found the expression of endoglin predominantly in the endothelium cover-
ing the atherosclerotic lesion, aortic valves, outside the lesion and in the capillaries of the surrounding myocardium in advanced atherosclerotic lesions in ApoE/LDLR double-knockout mice. We found only a weak expression of endoglin inside atherosclerotic plaque and almost no expression by smooth muscle cells in vessel media. These results are consistent with our previous studies of non-atherosclerotic vessels in normo- and hypercholesterolemic mice\(^7, 22\) suggesting that endoglin is expressed predominantly by the vessel endothelium in mice. This discrepancy with previous studies in humans might reflect the differences between human and mice atherogenesis.

Furthermore, it has been shown that endoglin regulates nitric oxide-dependent vasodilatation, as well as eNOS expression and activity\(^15, 23\). In this study, fluorescence immunohistochemistry revealed the colocalization of endoglin and eNOS in the aortic vessel endothelium covering atherosclerotic plaque only, suggesting the possible role of endoglin in the endothelium during atherogenesis. Moreover, recently, Santibanez et al. demonstrated that endoglin enhances eNOS expression by potentiating SMAD2 protein levels \textit{in vitro}, suggesting the role of endoglin in the regulation of eNOS expression\(^16\).

Endoglin forms complexes with TßRI and TßRII, and has been postulated to facilitate the binding of TGF-ß1 to these signaling receptors suggesting that endoglin affects TGF-ß signaling events\(^19\); however, increasing evidence also indicates that endoglin may have functions independent of TGF-ß1. For example, only about 1% of the endoglin molecules on endothelial cells bind TGF-ß, suggesting that endoglin has another, undefined physiological ligand\(^9\). Furthermore, it was demonstrated that endoglin upregulates eNOS expression at the transcriptional level, both in the absence and presence of exogenous TGF-ß in endothelial cells\(^25-27\).

We therefore focused on the endoglin relationship with SMAD proteins in our experiment. It was demonstrated that SMAD2 inhibits proinflammatory adhesion molecules, such as E-selectin, and at the same time induces eNOS expression\(^24, 28, 29\).

To the best of our knowledge, there are no \textit{in

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**Fig. 4.** Co-expression of endoglin and phosphorylated SMAD2/3 in the aorta of control ApoE/LDLR double-knockout mice.

Endoglin (green) and SMAD2/3 (red) co-expression was detected only in the aortic vessel endothelium covering atherosclerotic plaque (arrows). Strong phosphorylated SMAD2/3 staining is also visible in atherosclerotic intima and partially vessel media (arrowhead); however, no co-localization was detected in this area. Hoechst dye (blue) was used for counterstaining. Original magnification 100x.
**Endoglin Expression in Atherogenesis**

**vivo** studies showing that the above proteins are at least expressed simultaneously in the same cells in atherosclerotic lesions.

In this study, we demonstrated strong co-localization of endoglin with SMAD2 and phosphorylated SMAD2/3 in the aortic vessel endothelium. Although endoglin, SMAD2, and phosphorylated SMAD2/3 staining was detected even inside the atherosclerotic lesion, no co-localization of these proteins was found. Thus, here we demonstrate for the first time that endoglin, SMAD2, phosphorylated SMAD2/3, and eNOS are expressed together only by endothelial cells in vivo in advanced atherosclerotic lesions in mice, suggesting their possible role in vessel endothelium homeostasis and atherogenesis.

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

In contrast, in our previous study we found that atorvastatin decreased endoglin expression by both hypolipidemic and pleiotropic effects; however, there are some substantial differences when we compare with the results of this study. Firstly, the previous study used different mouse strains, apoE-deficient and C57BL/6J mice. Secondly, in the previous experiments we did not detect any atherosclerotic lesions in ApoE/LDLR double-knockout mice.

**Fig. 5.** Co-expression of endoglin and eNOS in the aorta of control ApoE/LDLR double-knockout mice.

Endoglin (green) and eNOS (red) co-expression was detected only in the aortic vessel endothelium covering atherosclerotic plaque (arrows). Hoechst dye (blue) was used for counter-staining. Original magnification 100x.
these mice and quantified the expression of endoglin in the intact non-atherosclerotic endothelium. We propose that the expression of endoglin might be regulated differently in intact non-atherosclerotic vessels when compared with vessels with advanced atherosclerotic lesions. Moreover, the difference in endoglin expression and quantity here and in previous studies could also be related to lipid metabolism. Initially,
cholesterol levels in this study with apoE/LDL receptor-deficient mice were markedly higher than previous experiments with C57BL/6J and apoE-deficient mice. Moreover, atorvastatin effects on endoglin expression could also be related to the presence of LDL receptor, which should be markedly increased after statin treatment. In this study, mice did not have the LDL receptor, which means that atorvastatin effects must be related to a different mechanism; however, we believe that the precise relationship between cholesterol levels and endoglin expression must be elucidated in a prospective in vitro study of endothelial cells (HUVEC).

The increase of eNOS expression after statin treatment was previously demonstrated and was related to a decrease in LDL and/or through statin inhibition of Rho geranylgeranylation through statins31, 32. It was also suggested that endoglin might be a proatherogenic marker participating in the development and progression of atherosclerosis3, 8, 20. In contrast, it could be proposed that the increase of eNOS expression in this study after atorvastatin treatment was related to the hypolipidemic effect of atorvastin shown previously18, which likely caused the upregulation of endoglin and SMAD2 expression in the vessel wall. This hypothesis is partially consistent with the very recent in vitro data of Chen et al. who clearly demonstrated that cholesterol suppresses and statins enhance SMAD2 phosphorylation25, 26; however, an in vitro mechanistic study elucidating the influence of cholesterol and/or statins on endoglin expression in endothelial cells is needed.

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

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