Cholesterol Effects on Endoglin and Its Downstream Pathways in ApoE/LDLR Double Knockout Mice

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Background: The aim of the study was to evaluate whether cholesterol-rich diet affects transforming growth factor-β-RIII (endoglin) levels in blood and 2 endoglin-related pathways in the aorta of ApoE/LDLR double knockout mice.

Methods and Results: Mice were fed either chow diet (CHOW) (n=8) or by 1% cholesterol-rich diet (CHOL) (n=8). Biochemical analysis of cholesterol and endoglin levels in blood, lesion size area, immunohistochemistry and Western blot analysis in mice aortas were performed. Biochemical analysis showed that cholesterol-rich diet resulted in a significant increase of cholesterol and endoglin levels in serum, and increased plaque size in the aorta. In addition, a cholesterol-rich diet significantly decreased the expressions of endoglin by 92%, activin receptor-like kinase (ALK)-1 by 71%, p-Smad2 by 21%, and vascular endothelial growth factor (VEGF) by 37% when compared to CHOW mice, but ALK-5, p-Smad1, and endothelial nitric oxide synthase were not significantly affected.

Conclusions: Hypercholesterolemia increases endoglin levels in blood and simultaneously decreases its expression in aorta, together with atherosclerosis protective markers p-Smad2 and VEGF, followed by increased plaque size. Inhibition of endoglin signaling might be one of the mechanisms responsible for the promoting of endothelial dysfunction and atherogenesis. Moreover, the monitoring of endoglin serum levels might represent an attractive blood marker of progression of disease; however, the precise source and role of endoglin in blood serum remains to be elucidated. (Circ J 2011; 75: 1747–1755)

Key Words: Activin receptor-like kinase; Atherogenesis; Endoglin; Smad proteins

Transforming growth factor-β (TGF-β) is a multifunctional growth factor that regulates the proliferation, differentiation, migration, extracellular matrix production, and survival of various cell types. TGF-β1 is expressed in a latent form, which first needs to be activated by proteases or thrombospondin before its binding to specific type I and type II serine/threonine kinase receptors. In TGF-β signaling, 1 TGF-β type II receptor (TGFβ/RII) and 2 distinct TGF-β type I receptors (ie, the endothelium restricted activin receptor-like kinase 1 (ALK1) and the broadly expressed ALK5) have been implicated.

Endoglin (CD105, TGF-βRII) is an auxiliary receptor of TGF-β signaling that plays a regulatory role in several TGF-β pathways. Endoglin associates with TGF-β/RII and modulates the activities of TGF-β/RII, ALK-1, ALK-5 and Smads in both in vitro and in vivo studies. In general, 2 functionally opposite pathways have been demonstrated. Endoglin/ALK-1/Smad1/5 promotes EC proliferation and migration, resulting, for example,
in angiogenesis. In contrast, the endoglin/ALK-5/Smad2/3 pathway leads to inhibition of ECs and quiescence of the endothelium.\textsuperscript{15,16}

The role of TGF-\(\beta\), endoglin, ALK-1, ALK-5 and various Smads in atherosclerosis has been studied in vivo in animals,\textsuperscript{19-21} and humans\textsuperscript{22-24} and in vitro,\textsuperscript{25-27} the results suggesting their participation in this disease. However, the precise role of endoglin and endoglin-related pathways in atherosclerosis are not known. Moreover, a detailed study of both the endoglin/ALK-5/Smad2 and endoglin/ALK-1/Smad1 pathway in atherosclerosis is not available currently.

Nitric oxide (NO) synthesis by endothelium is maintained by endothelial NO synthase (eNOS),\textsuperscript{30} which has been demonstrated to be affected by endoglin levels both in vitro and in vivo.\textsuperscript{26,29} Vascular endothelial growth factor (VEGF) is an important marker and strongly affects the function of endothelium, including the production of NO.\textsuperscript{30} It is also required for the maintenance of specialized EC stability and function and plays a considerable role in angiogenesis and atherosclerosis.\textsuperscript{31,32} Moreover, it was demonstrated that VEGF is related to the TGF-\(\beta\) signaling cascade and ALK-1 activity,\textsuperscript{33} and contributes to vascular stability by maintaining the endothelium in a quiescent state.\textsuperscript{34}

It has been previously demonstrated that cholesterol suppresses and modulates TGF-\(\beta\) signaling via the Smad2/3 pathway.\textsuperscript{35,36} Moreover, in our previous study, we showed that atorvastatin reduced cholesterol levels and increased the expression of endoglin/Smad2 and eNOS in the aorta of athero-sclerotic mice.\textsuperscript{3} In order to evaluate the effect of hypercholes-terolemia alone in vivo, we hypothesized whether cholesterol levels and the progression of atherosclerosis affect endoglin levels in blood and the 2 endoglin-related pathways, endoglin/ ALK-5/Smad2/eNOS and/or endoglin/ALK-1/Smad1/VEGF, in the aorta in ApoE/LDLR double knockout mice.

Methods

Animals

Two-month-old female ApoE/LDLR double knockout mice on a C57BL/6j background (n=16) (Jackson Laboratories, USA) were randomly subdivided into 2 groups.

All mice were fed with 2 different experimental diets for 2 months with water ad libitum throughout the study. The chow group (CHOW) of animals (n=8) was fed with chow diet. The cholesterol group (CHOL) of animals (n=8) was fed with a diet containing 1% of cholesterol.

Each mouse, in all groups, lived in a separate cage obtained 4 g of food (in specially prepared pellets) daily. Food consumption was monitored every day. No differences in the food consumption were visible, either between animals of one experimental group or between experimental groups.

At the end of the treatment period, all animals were fasted overnight and euthanased. The aortas, attached to the top half of the heart, were removed and then immersed in OCT embedding medium (Leica, Prague, Czech Republic), snap frozen in liquid nitrogen and stored at −80°C before histochemical and immunohistochemical staining. Descending aortas for western blot analysis were frozen in liquid nitrogen and stored at −80°C.

Biochemistry

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

ELISA Analysis
Endoglin levels in the serum of mice were measured by quantitative sandwich enzyme immunoassay technique using a commercial diagnostic kit, mouse Endoglin/CD105 Quan-tikine ELISA Kit (R&D Systems, MN, USA).

Oil Red Staining
Frozen sections were used for the detection of lipids in the atherosclerotic lesions. Slides were air-dried at room temperature for 30 min, stained in working solution of Oil Red for 30 min and counterstained with Gill’s hematoxylin.

Quantitative Analysis of the Oil Red Staining
Stereological methods for the estimation of lesion size area were performed by using stereological approach as described previously.\textsuperscript{37} In brief, the systematic uniform random sampling and the principle of the point-counting method were used for the estimation.\textsuperscript{37}

Immunohistochemistry
Sequential tissue sectioning started in the heart until the aortic root containing the semilunar valves together with the aorta appeared. From this point on, serial cross-sections (7 \(\mu\)m) were cut on a cryostat and placed on gelatin-coated slides. For the detection of endoglin expression the slides were in-cubated with anti-avidin and anti-biotin solutions (Vector Laboratories, USA). Thereafter, slides were incubated with primary antibodies for 1 h at room temperature and were de-veloped with biotin-conjugated goat anti-rat Ig (dilution 1:400 in bovine serum albumin) (Vector Laboratories) in the presence of 200 \(\mu\)g/ml normal mouse IgG (Dako, Denmark) afterwards. Antibody reactivity was detected using horseradish peroxidase-conjugated biotin–avidin complexes (Vector Laboratories) and developed with diaminobenzidine tetrahydro-chloride substrate (Dako).

Primary antibodies included the following: monoclonal antibody rat anti-mouse endoglin CD 105 (dilution 1:50) directed to the endoglin (BD Pharmingen, CA, USA); rabbit polyclonal antibody anti-ACVRL1/ALK-1 (dilution 1:100); anti-pSmad-1 (dilution 1:100); anti-VEGF (dilution 1:100, Abcam, Cambridge, UK); rabbit polyclonal anti-TGF-\(\beta\)-RI (ALK-5) (dilution 1:300, Sigma Chemical Co, St Louis, MO, USA), rabbit polyclonal anti-pSmad-2 (dilution 1:50, Millipore, Billerica, MA, USA), rabbit polyclonal anti-eNOS (dilution 1:50, Alexis Biochemicals, CA, USA).

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

Western Blot Analysis
Mouse aorta samples were homogenized in RIPA lysis buf-fer (Sigma Chemical Co) as described previously.\textsuperscript{38} Homog-
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enates (15 μg of aorta proteins) were used for membrane preparation as described previously. The membranes were blocked for 1h with 5% nonfat dry milk in Tris-buffered saline containing 0.05% Tween-20 (TBST), and then incubated with primary antibodies at the following concentrations: goat polyclonal antibody anti-endoglin directed to the endoglin (90–95 kDa) at 1:500, rabbit polyclonal anti-TGFβ-RI (ALK-5) directed to the TGFβ-RI (53 kDa) at 1:500, and anti-eNOS directed to the eNOS (140 kDa) at 1:200 (Santa Cruz Biotechnology, CA, USA), rabbit polyclonal antibody anti-ACVRL1/ALK-1 directed to the ACVRL1/ALK-1 (56 kDa) at 1:500, anti-p-Smad1 directed to the p-Smad1 (52 kDa) at 1:200, and anti-VEGF at 1:1,000 (Abcam, UK), rabbit polyclonal anti-p-Smad2 directed to the p-Smad2 (58 kDa) at 1:500 (Millipore, USA). As the loading control for western blotting, we used rabbit polyclonal anti-β-actin antibody (42–45 kDa) at 1:5,000 (Sigma Chemical Co), horseradish peroxidase conjugated rabbit anti-goat immunoglobulin G at 1:5,000 (Pierce Biotechnology, Rockford, IL, USA) and horseradish peroxidase-linked F(ab’)2 Fragment donkey anti-rabbit antibody at 1:2,000 (GE Healthcare, Prague, Czech Republic). The chemiluminescent process and quantification of immunoreactive bands on the exposed films were carried out as described previously. Equal loading of proteins onto the gel was confirmed by immunodetection of β-actin.

Statistical Analysis
All values in the graphs are presented as mean±SEM of 8 animals. Statistical significance in the differences between groups was assessed by t-test using GraphPad Prism 5.0 software (GraphPad Software, Inc, San Diego, CA, USA). P values of 0.05 or less were considered statistically significant.

Results
Biochemical Analysis of Cholesterol Levels in Mice
Biochemical analysis of blood samples of the ApoE/LDLR double knockout mice showed that the administration of 1%
cholesterol in diet resulted in a significant increase in total cholesterol (17±0.8 vs. 50±1.7 mmol/L, P<0.001), VLDL cholesterol (10±0.5 vs. 44±2.1 mmol/L, P<0.001) and LDL cholesterol (6±0.2 vs. 12±0.5 mmol/L, P<0.001) in comparison with CHOW mice (Figure 2). However, HDL cholesterol levels were unchanged by the cholesterol-rich diet when compared to CHOW mice (0.5±0.01 vs. 0.5±0.1 mmol/L). Moreover, triglycerides levels were lower after administration of the cholesterol-rich diet in comparison with CHOW mice (2.60±0.3 vs. ±1.01 mmol/L, P<0.01) (Figure 1A).

ELISA Analysis
The aim of the ELISA analysis was to reveal changes in the endoglin levels in serum in all mice. Mice that ate the cholesterol-rich diet had significantly higher endoglin levels in blood when compared with CHOW mice (1,669±314 vs. 2,601±272 pg/ml, P<0.05) (Figure 1B).

Atherosclerosis Lesion Size Quantification
The size of atherosclerotic lesions stained by Oil Red staining was quantified by stereological methods. Administration of cholesterol in the diet was associated with a larger Oil Red positive area in the aortic sinus when compared to CHOW mice (Figures 2A, B). Moreover, quantitative analysis indicated a significant increase of lesion size in cholesterol-fed mice in comparison with CHOW mice (0.2±0.01 vs. 0.3±0.01 mm², P<0.05) (Figure 2C).

Immunohistochemical Staining
All immunohistochemistry was performed in the aortic sinus of all mice. We focused on the expression of endoglin, ALK-1, ALK-5, p-Smad1, p-Smad2, VEGF and eNOS in atherosclerotic plaques, vessel media and intact endothelium in mice aortas. In general, the staining intensity was weak for all antibodies.

The expression of endoglin in the aortic sinus in both groups of mice was visible predominantly in endothelium covering the atherosclerotic lesion, endothelium of the aortic valves, outside the lesion and in the capillaries of the surrounding myocardium as described previously. Moreover, endoglin staining intensity was reduced in CHOL mice when compared with the CHOW mice (Figures 3A, B).

Similar staining patterns were detected for eNOS expression, which was also detected in endothelium only. However, the expression was generally weaker when compared to endoglin expression. In addition, no changes in eNOS expression were found in CHOL mice as compared to CHOW animals (data not shown).

ALK-1 and ALK-5 expressions were similar with respect to the areas of positivity. The expressions of ALK-1 and ALK-5 were found in vessel adventitia, media, atherosclerotic plaque and endothelium in all groups. Moreover, ALK-1 expression was weaker in CHOL mice when compared to CHOW mice (Figures 3C, D).

Phosphorylated forms of Smad1 and Smad2 were detected in the nuclei of cells. The expression was visible in adventitia, vessel media, atherosclerotic lesion and also in endothelium. p-Smad2 staining intensity was reduced in cholesterol-fed mice when compared with CHOW mice (Figures 3E, F). However, the cholesterol-rich diet did not affect the expression of p-Smad1 (data not shown). VEGF was also expressed in the whole vessel wall, including the endothelium, atherosclerotic plaque and vessel media. Furthermore, the cholesterol-rich diet resulted in decreased VEGF staining when compared to CHOW mice (Figures 3G, H).

Western Blot Analysis in Mice Aortas
Western blot analysis was performed in order to evaluate the changes in the expressions of endoglin, ALK-1, ALK-5, p-Smad1, p-Smad2, VEGF and eNOS in mice aortas.
Figure 3. Representative sections of endoglin (A, B), ALK-1 (C,D) p-Smad2 (E,F) and VEGF (G,H) expression in CHOW and CHOL mice. The expression of endoglin was detected only in endothelium (arrows). The expression of ALK-1, p-Smad2 and VEGF was visible in the whole vessel wall (arrows). Administration of cholesterol reduced the expressions of endoglin, ALK-1, p-Smad2 and VEGF when compared to CHOW mice. The slides were counterstained with hematoxylin. Scale bar = 100 μm.
Figure 4. Western blot analysis of endoglin (A), ALK-1 (B), ALK-5 (C), pSmad-1 (D), pSmad-2 (E), VEGF (F) and eNOS (G) in the aortas of CHOW and CHOL mice. The administration of a cholesterol-rich diet significantly decreased the levels of endoglin, ALK-1, p-Smad2, and VEGF in the aorta. On the other hand, the expressions of ALK-5, p-Smad1, and eNOS were not significantly changed after eating a cholesterol-rich diet (CHOL mice) when compared to CHOW mice. Equal loading of proteins onto gel was confirmed by immunodetection of β-actin (A). Values are expressed as means ± SEM of 6 measurements. **P<0.01, *P<0.05 vs. control. Top: densitometric analysis (control = 100%); Bottom: representative immunoblots. Equal loading protein was confirmed by β-actin.
The administration of a 1% cholesterol-rich diet significantly decreased endoglin expression by 92%, ALK-1 expression by 71%, p-Smad2 expression by 21%, and VEGF expression by 37% when compared to CHOW mice (Figures 4A, B, E, F, respectively). On the other hand, ALK-5, p-Smad1, and eNOS were not significantly affected by the feeding of a cholesterol-rich diet (Figures 4C, D, G, respectively). Equal loading of proteins onto gel was confirmed by immunodetection of β-actin as exemplified in Figure 4A.

**Discussion**

TGF-β signaling depends on the interplay between various TGF-β receptors and Smad proteins, which determine its action in various types of tissues. Moreover, it has been demonstrated that changes in TGF-β1 signaling represent a potential anti-atherosclerotic effect, which includes anti-inflammatory and plaque stabilizing effects. Moreover, it has been shown in vitro that cholesterol itself is able to modulate and suppress TGF-β1 signaling and the TGF-β1/RI/RII ratio. However, there are no data about cholesterol’s effects on TGF-β/R1I (endoglin) in vivo.

Several in vitro studies have demonstrated the crucial role of endoglin in TGF-β1 signaling in EC by potentiation of the ALK-1/Smad1 and inhibition of the ALK-5/Smad3 pathways and activation of ALK-5/Smad2 pathway. In order to evaluate the effect of hypercholesterolemia alone, we hypothesized whether cholesterol levels would affect endoglin levels in blood and endoglin-related pathways in the aorta in ApoE/LDLR double knockout mice. Administration of a 1% cholesterol-rich diet significantly increased both cholesterol level and the area of the atherosclerotic lesion when compared to animals fed chow diet only. Since it was demonstrated that endoglin may be released from the cellular membrane into the circulation as soluble endoglin, and that serum endoglin levels are elevated in atherosclerosis, we also evaluated the levels of endoglin in blood serum. Results of this current study revealed a significant increase in the endoglin serum levels in mice fed a cholesterol-rich diet when compared to mice fed chow diet only. This suggests that cholesterol levels might affect and/or be related to endoglin levels in serum, which is line with previously published data that cholesterol levels correlate with endoglin levels in blood serum. Those authors suggested that increased serum levels of endoglin might be a marker of progression of atherosclerosis. On the other hand, the major source of soluble endoglin in atherosclerosis is not known, so further analysis is necessary in order to elucidate the precise role of endoglin serum level changes in atherosclerosis. In order to elucidate how endoglin levels in blood and tissues are related, we evaluated the expression of endoglin and the members of its pathway in mice aortas.

Many in vitro studies have demonstrated that endoglin affects TGF-β signaling by modulating ALK and Smad activity. In general, recent studies have revealed an intricate interplay between the 2 signaling pathways and endoglin. Endoglin/ALK-1/Smad1/5 signaling has been shown to stimulate EC migration, proliferation and tube formation, resulting in increased angiogenesis. In contrast, activation of the endoglin/ALK-5/Smad2/3 pathway inhibits the activity of ECs, blocks angiogenesis by inhibiting EC proliferation, tube formation and migration, and keeps the endothelium quiescent. Moreover, ALK5/Smad2/3 inhibits ALK1-Smad1/5 signaling and vice versa, and ALK5 is required for efficient TGF-β/ALK1 signaling.

In this study, the cholesterol-rich diet resulted in a significant decrease in endoglin, ALK-1, p-Smad2 and VEGF expressions in aorta. However, a previous study in humans demonstrated that endoglin, TGF-β1 and TGF-β2 are expressed in atherosclerotic vessels, whereas expression is low in non-atherosclerotic vessels, suggesting the participation of endoglin in the atherosclerotic process. The expression of endoglin in humans was detected in ECs, macrophages and smooth muscle cells, whereas the expression of endoglin was detected only in the endothelium of the mice in our study. In addition, Piao et al compared non-atherosclerotic vessels with atherosclerotic vessels, but that does not rule out the possibility of endoglin expression changing during the progression of atherosclerosis, which was demonstrated in our study. Thus, the results of our study in mice are partially in line with recently published results showing that cholesterol is able to decrease the expression of TGF-β1/RII and p-Smad2 in the aorta of apoE-deficient mice compared to wild-type mice. Chen et al also showed that TGF-β-R1I expression did not differ between apoE-deficient and wild-type mice. However, in our study only one subtype of TGF-β-R1I (ALK-5) did not change, while the other subtype, ALK-1 was decreased after cholesterol-rich diet. Moreover, other members of the endoglin cascade, p-Smad1 and eNOS, were also not affected by the cholesterol-rich diet. As mentioned before, it has been demonstrated that endoglin regulates the signal transduction of TGF-β1 by potentiation of the ALK1/Smad1 pathway, and also by enhancing ALK5/Smad2 signaling. Therefore, it is surprising that in this study ALK-5 (a member of the endoglin/ALK-5/Smad2 pathway) expression was not affected by cholesterol level or lesion size, whereas endoglin and p-Smad2 were. This discrepancy can be explained by reference to previously published in vitro data where endoglin was shown to actually increase Smad2 expression directly without ALK-5. The decrease in the endoglin/p-Smad2 levels in mice might be considered as a pro-atherogenic effect since it was demonstrated that activation of endoglin/p-Smad2 signaling has plaque-stabilizing effects in humans; however, this was not analyzed in this study. Surprisingly, the expression of eNOS was not affected by the cholesterol-rich diet in this study, despite the fact that previous studies clearly demonstrated a relation between endoglin/Smad2 signaling and eNOS levels. This suggests that eNOS expression is not exclusively regulated by endoglin/Smad2 signaling. On the other hand, it does not rule out the possibility that the production of NO is affected by the administration of cholesterol.

VEGF is a pleiotropic growth factor that seems to play a role in atherogenesis. It has been shown that VEGF can participate in the progression of atherosclerosis by promoting macrophage chemotaxis. However, its activity is strongly related to plaque neovascularization, increasing vessel permeability and promoting of inflammatory cells infiltration via new vessels into atherosclerotic plaque. Nevertheless, these effects of VEGF are unlikely to have been manifested in this study, because neither angiogenesis nor neovascularization was detected in the vessel wall. In addition, it has been demonstrated that VEGF is required for EC stability, inhibition of rolling of leukocytes, and that it possess anti-atherosclerotic and anti-apoptotic effects. Moreover, VEGF stimulates production of NO. In addition, VEGF expression is increased by ALK-1 in vitro. In this study, we demonstrated that cholesterol administration reduces endoglin/ALK-1/VEGF expression in the murine aorta and increases atherosclerotic plaque size, which suggests that cholesterol might affect NO production by affecting this pathway and thus
partially contribute to theatherogenic process.

It is also of interest that endoglin levels were increased in blood after administration of the cholesterol diet and simultaneously decreased in the vessel wall. This discrepancy could reflect the previously published data where increased serum levels of endoglin were related to its decreased expression and signaling in tissue followed by manifestation of endothelial dysfunction, and preeclampsia.

**Conclusion**

We have shown for the first time that hypercholesterolemia increases endoglin levels in blood and simultaneously decreases its expression in the aorta, together with atherosclerosis protective markers p-Smad2 and VEGF, which was followed by an increase in plaque size. We propose that inhibition of endoglin signaling might represent one of the mechanisms responsible for the promoting of endothelial dysfunction and atherogenesis. Moreover, the monitoring of endoglin serum levels might represent an attractive blood marker of progression of disease; however, the precise source and role of endoglin in blood serum remains to be elucidated.

**Acknowledgments**

The authors thank Gwawr Edwards and Eleanor Martin for their help with the immunohistochemical staining and the manuscript. This work was supported by grants from The Grant Agency of Charles University in Prague, numbers 129208/C and 137310/C, grant MSM 002162082, grant SVV/2011/263-003 and Research project MZO 00179906.

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