Effect of lipanthyly on mRNA expression of endothelin-1 and nitric-oxide synthase in atherosclerotic vessel wall in rabbits

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ABSTRACT

AIM. To study the mechanism of regression of atherosclerosis AS by lipanthyly. METHODS. Experimental atherosclerotic rabbits created by damaging the abdominal aortic endothelium and feeding with high fat diet for 8 wk were then treated with lipanthyly 15 mg kg\(^{-1}\) d\(^{-1}\) for 16 wk. Expression of endothelin-1 ET-1 mRNA and nitric oxide synthase NOS mRNA in atherosclerotic vessel wall was measured by in situ hybridization and reverse transcription polymerase chain reaction RT-PCR respectively. RESULTS. After lipanthyly administration for 16 wk ET-1 mRNA expression was reduced and integral optical density IOD and area of hybridization granule were observed to be 49 113 ± 16 864 and 2448 ± 621 \(\mu m^2\) in lipanthyly group and 65 183 ± 10 113 and 3028 ± 352 \(\mu m^2\) in atherosclerotic group respectively. Regarding inducible NOS mRNA expression IOD and area were decreased by 25.5 % and 53.3 % respectively whereas endothelial NOS mRNA expression was increased. CONCLUSION. Restoration of the disturbed ET-1 mRNA/NOS mRNA balance by lipanthyly might be one of its mechanisms leading to regression of atherosclerosis.

INTRODUCTION

The increase in blood lipid and the interaction of vessel wall cells with their cytokines plays a marked role in the process of atherosclerotic generation and development. The predominant molecular form of endothelin ET-1 in hypercholesterolemia was the biologically active ET-1, it also involved in atherosclerosis AS formation including vascular intimal lesion monocyte infiltration low density lipoprotein LDL oxidative modification and smooth muscle cell proliferation etc. Whereas nitric oxide NO might inhibit all key processes participating in the early pathogenesis of AS such as monocyte and leucocyte adhesion platelet-vascular wall interaction vascular smooth muscle cell proliferation and migration. Recent studies suggest that lipid-lowering therapy might have a beneficial effect on the regression of AS. Lipanthyly a 3-hydroxy-3-methylglutaryl-coenzyme A HMGCoA reductase inhibitor can inhibit the intracellular cholesterol biosynthesis and remove LDL from the circulation. To investigate the possible mechanism of regression of AS we observed the effect of the drug on mRNA expression of ET-1 and nitric-oxide synthase in atherosclerotic vessel wall of rabbits.

MATERIAL AND METHODS

Rabbits Male New Zealand White rabbits weighing 2 – 2.5 kg were obtained from Animal Breeding Center of Suzhou Medical College.

Reagents Lipanthyly was procured from Laboratoires Fournier SA. France. Dig DNA labeling and detection kit and titanTM one tube RT-PCR system were products of Boehringer Mannheim. Trizol was a product of Gibco/BRL. Probes of ET-1 cDNA and inducible NOS eNOS primers were synthesized by Institute of Microbiology, Chinese Academy of Sciences. GAPDH primers were kindly provided by Prof Tang Jian of Beijing Medical University. Other chemi-
Preparation of atherosclerotic model Experimental atherosclerotic model was created in a 10-cm segment of the abdominal artery of rabbits by damaging the endothelium using 4F Forgarty embolus catheter (Biosensors International Pte Ltd) and feeding the rabbits a high fat diet (cholesterol 0.5 g kg\(^{-1}\), d\(^{-1}\) and lard 0.5 mL kg\(^{-1}\) d\(^{-1}\)) for 8 wk. After the diet was terminated, the rabbits were randomized to either an atherosclerotic group [n = 6] or an experimental group [n = 6]. The former was fed on the routine diet whereas the latter was fed on routine diet supplemented with lipanthyl 15 mg kg\(^{-1}\) d\(^{-1}\). A control group [n = 6] was added additionally. The rabbits were then killed 16 wk after administration of the drug and the abdominal artery was dissected and a portion of it was fixed in 10% formalin for in situ hybridization; the rest was stored at liquid nitrogen for total RNA extraction.

In situ hybridization Sections of paraffin embedded tissues were deparaffinized and rehydrated according to standard protocols then treated with protein K 1 mg/L for 15 min at 37 °C and fixed with 4% paraformaldehyde for 10 min at room temperature. Sections were preincubated for 30 min at 42°C in a solution containing 6 x sodium chloride 0.9 mol L\(^{-1}\][NaCl] and sodium citrate 90 mmol L\(^{-1}\][buffer][SSC] 45% formamide 5 x Denhardt’s solution and denatured salmon sperm DNA 100 mg L\(^{-1}\] and incubated for 20 h at 42°C with 50 μL of the same solution with denatured probes 1 mg L\(^{-1}\]. The tissue sections were then rinsed in 6 x SSC-45% formamide 2 x SSC buffer 1 x maleic acid 100 mmol L\(^{-1}\] NaCl 0.15 mol L\(^{-1}\] pH 7.5[buffer][SSC] 9 mL[buffer] blocking solution 1 mL[buffer] respectively covered with anti-digoxigenin antibody conjugated with alkaline phosphatase[buffer] diluted 1:250 in buffer 1 for 30 min at room temperature[buffer] rinsed in buffer 1 twice and then substrate solution. NBT/BCIP was added in the dark and the tissue sections were kept for 6 h. The reaction was terminated by washing in a buffer containing Tris 10 mmol L\(^{-1}\] and edetic acid 1 mmol L\(^{-1}\].

Reverse transcription polymerase chain reaction (RT-PCR) The total RNA of vessel wall tissue was isolated using trizol reagent and the final RNA pellet was redissolved in 0.1% diethylpyrocarbonate water and stored at -70 °C the RNA was quantified by measuring the A\(_{260}\]. Subsequent RT-PCR was performed in DNA thermal cycle PE-2000 USA according to the operating manual of titam™ one tube RT-PCR system. For amplification of the desired cDNA the eNOS primer sense 5’-GGT GAA TTC AT ACAA GCC TGC TCG ATG GAA CAC-3’ antisense 5’-GGT AAG CTT CTCT CCT GTC CGC AAA GCT CAT-3’ were used. The RT was carried out at 50 °C for 30 min and the cycle program was set to denature at 94 °C for 45 s to anneal at 58 °C for 45 s and to extend at 72 °C for 1 min for a total of 33 cycles. In the end a prolonged elongation time of 5 min at 72 °C was given. The PCR products were clearly visible after 1.5% agarose gel electrophoresis and ethidium bromide staining.

Statistical analysis Integral optical density IOD and area of hybridization granule for ET-1 mRNA and iNOS mRNA were determined by the technique of image pattern analysis. The data are express as \(x \pm s\) and \(t\) test was used for intragroup comparisons.

RESULTS

ET-1 and iNOS in situ hybridization IOD and area of hybridization granule for ET-1 mRNA and iNOS mRNA in atherosclerotic model were significantly increased as compared with control group [P < 0.01 two-sided test]. After treated with lipanthyl 15 mg kg\(^{-1}\), d\(^{-1}\) for 16 wk ET-1 mRNA expression was decreased by 19.2% IOD - 24.6% area [P < 0.05 one-sided test] and iNOS mRNA expression was also reduced by 25.5% IOD - 53.3% area [P < 0.01 two-sided test] Tab 1.

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<th>ET-1 mRNA</th>
<th>iNOS mRNA</th>
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<tr>
<td></td>
<td>area/μm²</td>
<td>area/μm²</td>
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<tr>
<td>Normal</td>
<td>33477 ± 10633</td>
<td>3180 ± 1014</td>
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<tr>
<td>Atherosclerotic model</td>
<td>65188 ± 10113°</td>
<td>9701 ± 287°</td>
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<tr>
<td>Lipanthyl</td>
<td>49113 ± 16968°</td>
<td>7227 ± 1860°</td>
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Tab 1. Effects of lipanthyl on ET-1 mRNA and iNOS mRNA expression in atherosclerotic vessel wall in rabbits. \(n = 6, \ x \pm s\). \*P < 0.01 vs normal. \*P < 0.05 \*P < 0.01 vs atherosclerotic model.
eNOS RT-PCR  The PCR products had a length of 661 bp. In equal templates eNOS mRNA expression in atherosclerotic aorta seemed to be diminished in atherosclerotic group. Treatment of rabbits with lipanthy 15 mg kg−1 d−1 for 16 wk resulted in an increase in eNOS mRNA expression Fig 1.

![Image](image)

**Fig 1.** Effects of lipanthy on expression of eNOS mRNA in rabbit atherosclerotic abdominal aorta. M PCR marked A normal B atherosclerotic model C lipanthy.

**DISCUSSION**

The present results show that expression of ET-1 mRNA and iNOS mRNA in atherosclerotic vessel wall is significantly increased whereas expression of eNOS mRNA is decreased. These results are in accordance with previous observations. It suggests that the balance between ET-1 mRNA and NOS mRNA is disrupted at the transcriptional level in the atherosclerotic vessel wall.

We have demonstrated that lipanthy caused regression of AS but its mechanism was not clear. Lipanthy is a HMGCoA reductase inhibitor and can inhibit the intracellular cholesterol biosynthesis. It is believed that HMGCoA reductase inhibitor can prevent cell proliferation in response to mitogenic stimuli. In our studies we found that the increase of ET-1 mRNA expression in rabbit atherosclerotic abdominal aorta decreased after administration of lipanthy 15 mg kg−1 d−1 for 16 wk as this might have resulted in the reduction of ET-1 in vessel wall.

Increase in NO production may block progression of AS but NO in the form of iNOS is implicated in the pathophysiological processes due to the formation of peroxynitrite ONOO−. Therefore it is now well accepted that increase in iNOS activity and decrease in eNOS activity may exacerbate the AS. Our result showed that lipanthy might reduce iNOS mRNA expression and enhance eNOS mRNA expression in atherosclerotic vessel wall thus it revealed that the drug might abate the atherosclerotic degree by regulating the expression of NOS subgroup genes.

In conclusion lipanthy not only reduced blood lipids but also regulated the mRNA expression of ET-1 and NOS in atherosclerotic vessel wall which might be one of its mechanisms causing regression of AS.

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