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

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KEY WORDS rabbit；lipanthyl；endothelin-1；nitric-oxide synthase；gene expression；atherosclerosis

ABSTRACT

AIM：To study the mechanism of regression of atherosclerosis (AS) by lipanthyl. **METHODS**：Experimental atherosclerotic rabbits created by damaging the abdominal aortic endothelium and feeding with high fat diet for 8 wk were then treated with lipanthyl 15 mg·kg^{-1}·d^{-1} for 16 wk. Expression of endothelin (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 lipanthyl 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 ± 16 868) and (2448 ± 621) μm^2 in lipanthyl group and (65 188 ± 10 113) and (3028 ± 352) μm^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 lipanthyl 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[1-3]. The predominant molecular form of endothelin (ET) in hypercholesterolemia was the biologically active ET-1[4], it also involved in atherosclerosis (AS) formation[5-7], including vascular intimal lesion, monocytic 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[8], platelet-vessel wall interaction[9], vascular smooth muscle cell proliferation and migration[10]. Recent studies suggest that lipid-lowering therapy might have a beneficial effect on the regression of AS[11-13]. Lipanthyl, 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 (NOS) 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 Lipanthyl was procured from Laboratoires Fournier SA (France). Dig DNA labeling and detection kit and titam^{TM} one tube RT-PCR system were products of Boehringer Mannhein. Trizol was a product of Gibco/BRL. Probes of ET-1 cDNA and inducible NOS (iNOS) cDNA were supplied by Zhongshan Biological Technology Co Ltd (Beijing, China). Endothelial NOS (eNOS) primers were synthesized by Institute of Microbiology, Chinese Academy of Sciences (Beijing, China). GAPDH primers were kindly provided by Prof TANG Jian of Beijing Medical University. Other chemi-

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Received 1999-06-15 Accepted 1999-11-15

ISSN 0253-9756 Acta Pharmacol Sin 中国药理学报 2000 May；21 (5)
E-mail aps@mail.shcnc.ac.cn Phn/Fax 86-21-6474-2629

cals were of AR grade.

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 embolectomy catheter (Biosensors International Pte Ltd) and feeding the rabbits a high fat diet (cholesterol 0.5 g·kg^{-1}·d^{-1}, 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, 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 deparaffinated and rehydrated according to standard protocols, then treated with protein K 1 mg/L for 15 min at 37 ℃ and fixed with 4 % paraformaldehyde for 10 min at room temperature. Sections were preincubated for 30 min at 42 ℃ in a solution containing 6 × sodium chloride (0.9 mol·L^{-1}) and sodium citrate (90 mmol·L^{-1}) buffer (SSC), 45 % formamide, 5 × Denhardt's solution and denatured salmon sperm DNA 100 mg·L^{-1}and incubated for 20 h at 42 ℃ with 50 μL of the same solution with denatured probes 1 mg·L^{-1}. The tissue sections were then rinsed in 6 × SSC-45 % formamide, 2 × SSC, buffer Ⅰ (maleic acid 100 mmol·L^{-1}, NaCl 0.15 mol·L^{-1}, pH 7.5), and buffer Ⅱ (buffer Ⅰ 9 mL, blocking solution 1 mL), respectively, covered with anti-digoxigenin antibody conjugated with alkaline phosphatase (diluted 1∶250 in buffer Ⅰ) for 30 min at room temperature, rinsed in buffer Ⅰ twice, and then substrate solution (NBT/BCIP) was added in the dark and the tissie 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 ℃, the RNA was quantified by measuring the A_{260}. Subsequent RT-PCR was performed in DNA thermal cycler (PE-200, USA) according to the operating manual of titam™ one tube RT-PCR system. For amplification of the desired cDNA, the eNOS primers (sense: 5′-GGT GAA TTC ATA CCA GCC TGA TCC ATG GAA CAC-3′, antisense: 5′-GGT AAG CTT CTT CTT CCT GTC CGC AAA GCT CAT-3′) were used. The RT was carried out at 50 ℃ for 30 min, and the cycle program was set to denature at 94 ℃ for 45 s to anneal at 58 ℃ for 45 s, and to extend at 72 ℃ for 1 min for a total of 33 cycles. In the end, a prolonged elongation time of 5 min at 72 ℃ 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 $\bar{x} \pm s$, 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).

Tab 1. Effects of lipanthyl on ET-1 mRNA and iNOS mRNA expression in atherosclerotic vessel wall in rabbits. $n = 6$. $\bar{x} \pm s$. $^{c}P < 0.01$ *vs* normal. $^{e}P < 0.05$, $^{f}P < 0.01$ *vs* atherosclerotic model.

	ET-1 mRNA		iNOS mRNA	
	IOD	area/μm^2	IOD	area/μm^2
Normal	38477 ± 10633	1930 ± 355	3180 ± 1014	22 ± 4
Atheosclerotic model	65188 ± 10113^{c}	3028 ± 352^{c}	9701 ± 287^{c}	92 ± 12^{c}
Lipanthyl	49113 ± 16868^{e}	2448 ± 621^{e}	7227 ± 1868^{f}	43 ± 4^{f}

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 lipanthyl 15 mg·kg^{-1}·d^{-1} for 16 wk, resulted in an increase in eNOS mRNA expression (Fig 1).

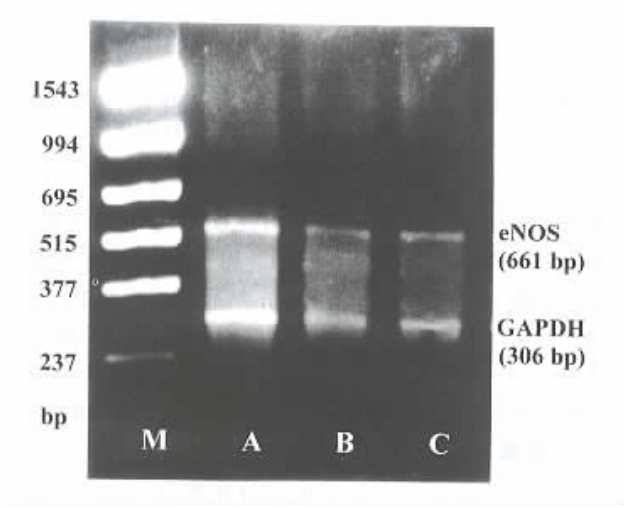

Fig 1. Effects of lipanthyl on expression of eNOS mRNA in rabbit atherosclerotic abdominal aorta. M：PCR marker；A：normal；B：atherosclerotic model；C：lipanthyl.

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[14]. 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 lipanthyl caused regression of AS, but its mechanism was not clear. Lipanthyl 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[15]. In our studies, we found that the increase of ET-1 mRNA expression in rabbit atherosclerotic abdominal aorta decreased after administeration of lipanthyl 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[16,17]. Our result showed that lipanthyl 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, lipanthyl 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.

ACKNOWLEDGMENTS We thank LU Qun and ZHU Lu-Jia for their technical assistance.

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ISSN 0253-9756 Acta Pharmacol Sin 中国药理学报 2000 May；21 (5)
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力平脂对兔粥样硬化血管壁组织内内皮素-1 和一氧化氮合酶 mRNA 表达的影响[1]

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关键词 兔；力平脂；内皮素-1；一氧化氮合酶；基因表达；动脉粥样硬化

目的：探讨力平脂消退动脉粥样硬化的作用机制. 方法：给兔喂饲高脂饲料 8 周，加腹主动脉内皮损伤造成腹主动脉粥样硬化模型，然后用力平脂 15 mg·kg^{-1}·d^{-1}治疗 16 周. 粥样硬化血管壁组织中内皮素(ET)-1 和一氧化氮合酶(NOS) mRNA 的表达分别采用原位杂交和逆转录多聚酶链反应(RT-PCR)测定. 结果：力平脂治疗 16 周后，ET-1 mRNA 表达明显减少，其阳性杂交颗粒的积分光密度(IOD)和面积分别由粥样硬化组的(65 188 ± 10 113)和(3028 ± 352) μm^2 下降至(49 113 ± 16 868)和(2448 ± 621) μm^2. 力平脂也可降低诱导型 NOS mRNA 的表达，使 IOD 和面积分别下降 25.5 % 和 53.3 %，但通过 RT-PCR 测得的内皮型 NOS mRNA 表达增加. 结论：恢复 ET-1 mRNA/NOS mRNA 的平衡可能是力平脂消退动脉粥样硬化的机制之一.

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