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*Research Paper*

# Effects of simvastatin on early oxidative stress and endothelial function in apolipoprotein E-deficient mice

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## Abstract

**Objective:** To investigate the mechanisms that Simvastatin, a 3-hydroxy-3-methylglutaryl coenzyme A(HMG-CoA) reductase inhibitor, plays an important role in primary prevention of atherosclerosis independently of its lipid-lowering effect in Apolipoprotein E-deficient mice in the early stage of atherosclerosis. **Methods:** Twenty-four 6-week old male apoE-deficient mice were randomly divided into two groups:control group(normal saline) and treatment group[simvastatin(5 mg/(kg • d))]. Simvastatin was administered to treatment group mice by gavage and the same volume of normal saline was administered to control group mice by the same method for 2 or 4 weeks. Total cholesterol(TC), super-oxide dismutase(SOD), malondialdehyde(MDA) and serum nitric oxide(NO) were measured by biochemical analysis. **Results:** There was no significant difference in serum TC between control and treatment groups. Compared with the control's, the effects of simvastatin were more significant in decreasing serum MDA level($P < 0.01$ vs control's at 2-week; $P < 0.006$ vs control's at 4-week), increasing serum SOD level($P < 0.03$ vs control's at 2-week; $P < 0.003$ vs control's at 4-week) and NO level ($P < 0.01$ control's at 2-week; $P < 0.001$ vs control's at 4-week) either at 2 or 4 weeks. **Conclusion:** Simvastatin attenuates oxidative stress and protects endothelial function by the mechanisms of decreasing serum MDA level, increasing serum SOD level and NO level , which were inconsistent with its cholesterol-lowering effect. It may play an important role in primary(if not all) prevention of atherosclerosis and might be independent of lipid-regulation mechanism.

*Key words*: simvastatin; apolipoprotein E-deficient mice; oxidative stress; nitric oxide

## INTRODUCTION

The previous data has demonstrated that the major effect of the 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors(simvastatin, pravastatin, etc., known as "tatins") is by the inhi-bition of cholesterol synthesis in liver. Recently, Vaughan's study[1-2] suggested simvastatin had effects of stabilizing plaque and decreasing oxidative stress on advanced atherosclerotic lesions. Zhang's results[3-4] showed pravastatin may play an important independent of lipid-regulation role in monocytes adhesion to endothelium in advanced atherosclerotic apolipoprotein E-Deficient mice. It has been proven that simvastatin has the effects independent of the lipid-regulation mechanism such as anti-inflammation, improving endothelial function, stabilizing plaque and so on[1-2]. This increasing evidence[4-7,15-16] strongly suggested that statins could improve endothelial function and protect the cardiovascular system independent of their cholesterol-lowering effects. Oxidative stress is the first step in the atherosclerotic progress. Cardiovascular risk-factors can cause oxidative stress-reactive oxygen species(ROS) increasing in the vessel wall. Superoxide dismutase(SOD) and malondialdehyde(MDA) are markers of oxidative stress and determining serum SOD and MDA levels can reflect oxidative stress taken place in the endothelium[8]. Nitric oxide(NO) is endothelial-

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derived and its function-dependent marker. However, rare data show that statin therapy can exert anti-atherosclerosis effect in apolipoprotein E-deficient mice in the early stage of atherosclerosis when its serum cholesterol level has not markedly risen up. In the present study, we used Apolipoprotein E-deficient mice, a well-established genetic mouse model of atherogenic hypercholesterolemia, to test the hypothesis that simvastatin can decrease oxidative stress and protect endothelial function in the early stage of atherosclerosis. The study was meticulously designed so that blood cholesterol levels were not affected by statin treatment.

## METHODS

This study was performed in accordance with the Health guidelines of Animal Care and Use Committee of Nanjing Medical University for the use of experimental animals. All animal protocols were approved by the Nanjing Medical University Animal Care.

### Animals

Male apoE-deficient mice on C57BL/6J background, 6-weeks of age($n$ = 24, Animal Center of Beijing University), were fed with a chow diet throughout the study and randomly divided into two groups:control group(normal saline) and treatment group(simvastatin obtained from Merck Inc). Simvastatin[5 mg/(kg • d)] was administered to treatment group mice by gavage and the same volume of normal saline was administered to control group mice by the same method for 2 or 4 weeks. The dose of simvastatin used in the study was based on that used in previous studies with hyperlipidemia mice[8-10].

### Animal euthanasia and serum preparation

The mice were sedated(1% butaylone 0.5~1.0 ml), blood was collected from fossa orbitalis, and the animals were euthanized by exsanguinations after simvastatin treatment 0,2,4 weeks separately.

### Determination of TC

TC was determined by automatic biochemistry analyzer(Italian, SABA218), and quality control(QC) serum was added.

### Quantification of SOD, MAD, and NO

Serum SOD was measured by xanthine oxidase method; MAD was determined by barbituric acid method(TBA); $NO^{3-}$and $NO^{2-}$ were valued by nitrate reductase method , and total amount of $NO^{3-}$ and $NO^{2-}$ represented the NO levels. All kits were offered by Nanjing Jiancheng Biotechnology Company.

### Statistics analysis

The data were analyzed by STATA9.0 Statistics software. All data were presented as mean ± $s$. Data on mean TC, SOD, MDA and NO were compared by the Student 2-tailed t test, and probabilities of 0.05 or less were considered significant.

## RESULTS

### Effects of simvastatin on serum TC levels

Serum TC was unaffected by 2 or 4 weeks of simvastatin treatment(***Tab 1***). There was no significant difference between treatment and control groups either at 2 or 4 weeks.

***Tab 1*** **Effects of simvastatin on serum TC levels** (mmol/L, $\bar{x} \pm s$)

| | 0-week(mmol/L) | 2-week(mmol/L) | 4-week(mmol/L) |
|---|---|---|---|
| Control group ($n$ = 12) | 10.108 9 ± 1.475 6 | 11.775 7 ± 0.946 4 | 10.801 4 ± 2.098 4 |
| Simvastatin group ($n$ = 12) | 11.444 5 ± 1.013 3 | 12.632 6 ± 0.518 2 | 11.896 3 ± 0.203 6 |

### Effects of simvastatin on serum SOD levels

There was no significant difference between control and simvastatin groups at 0-week(***Tab 2***). Simvastatin group mice exhibited elevated serum SOD levels of (110.928 2 ± 5.202 7)U/L and (135.389 7 ± 5.566 4)U/L compared with that of control group(98.549 7 ± 4.054 0) U/L; $P$<0.03 and(97.286 6 ± 7.641 4)U/L; $P$<0.003) at 2 weeks or 4 weeks respectively. Administration of simvastatin significantly increased serum SOD levels.

***Tab 2*** **Effects of simvastatin on serum SOD levels** (U/L, $\bar{x} \pm s$)

| | 0-week(U/L) | 2-week(U/L) | 4-week(U/L) |
|---|---|---|---|
| Control group ($n$ = 12) | 110.993 2 ± 3.734 8 | 98.549 7 ± 4.054 0 | 97.286 6 ± 7.641 4 |
| Simvastatin group ($n$ = 12) | 100.395 9 ± 9.155 8 | 110.928 2 ± 5.202 7* | 135.389 7 ± 5.566 4# |

Compared with control group, *$P$< 0.03; Compared with control group, #$P$< 0.003.

### Effects of simvastatin on serum MDA levels

Serum MDA levels were similar in both control and treatment groups at the beginning of the study. However simvastatin markedly attenuated serum MDA levels[(12.000 0 ± 0.565 0)nmol/L vs(14.794 6 ± 0.854 4) nmol/L; $P$<0.01] at 2 weeks and[(10.564 1 ± 0.594 1)

nmol/L vs control's(17.384 6 ± 1.056 2)nmol/L; $P$ < 0.006) at 4 weeks](***Tab 3***). Administration of simvastatin significantly decreased serum MDA levels.

***Tab 3*** **Effects of simvastatin on blood MDA level** (mmol/L, $\bar{x} \pm s$)

| | 0-week(mmol/L) | 2-week(mmol/L) | 4-week(mmol/L) |
|---|---|---|---|
| Ontrol group ($n$ = 12) | 14.589 8 ± 1.064 3 | 14.794 6 ± 0.854 4 | 17.384 6 ± 1.056 2 |
| Simvastatin group ($n$ = 12) | 14.318 3 ± 0.719 0 | 12.000 0 ± 0.565 0* | 10.564 1 ± 0.594 1# |

### Effects of Simvastatin on Serum NO Levels

Mean serum NO levels in both control group and simvastatin group did not differ significantly at the beginning of the study(***Tab 4***). During the study, serum NO levels were significantly reduced in both control group[(22.759 9 ± 2.841 3) μmol/L; (12.365 6 ± 2.181 6 μmol/L) ] and treatment group[(37.278 2 ± 4.203 3) μmol/L; (28.494 6 ± 4.152 9) μmol/L] at 2 weeks or 4 weeks respectively. But serum NO levels of treatment group were much less reduced than control group either at 2 weeks or 4 weeks($P$ < 0.01 or $P$ < 0.001; ***Tab 4***). Treatment with simvastatin significantly reduced the extent of NO levels decreased in apolipoprotein E-deficient mice.

***Tab 4*** **Effects of simvastatin on blood NO levels** (μmol/L, $\bar{x} \pm s$)

| | 0-week | 2-week | 4-week |
|---|---|---|---|
| Control group ($n$ = 12) | 58.422 9 ± 2.361 5 | 22.759 9 ± 2.841 3 | 12.365 6 ± 2.181 6 |
| Simvastatin group ($n$ = 12) | 49.9105 ± 5.329 7 | 37.278 2 ± 4.203 3* | 28.494 6 ± 4.152 9# |

Compared with control group, *$P$ < 0.01; Compared with control group, #$P$ < 0.001.

## DISCUSSION

In 1992, there were two laboratories had reproduced apolipoprotein E-deficient mouse successfully by gene knock-out technology. The mouse had spontaneous hyperlipidemia because of clearing lipoprotein in lower speed, and its cholesterol level may achieve 1 113-1 515 mmol/L at normal diet .The nature and region of the mouse's atherosclerosis were similar to mankind's. Its diseased region would be larger and its progression would be more rapid if given hypercholesterol diet. So, it was an excellent animal model to study atheromatosis[11-12]. Studies showed that monocyte had attached to impaired endothelial cell at 5-6 weeks age , appeared fatty streak at 8-10 weeks age and formed fibrous plaque gradually after 15-20 weeks age in apolipoprotein E-deficient mice. The most obviously diseased region was in aortic root, then aorta thoracalis[9].

Oxidative stress is the earliest event occurring during the atherosclerotic progress, and it ultimately contributes to endothelial cell dysfunction. The endothelial dysfunction is characterized by marked reduction in NO and SOD, but augmentation in MDA. Recently, statins were reported to directly increase NO and SOD levels, decrease MDA level in advanced atherosclerotic Apolipoprotein E-deficient mice, and this effect of statins was associated with the inhibition of oxidative Stress.

Cardiovascular risk-factors(hyperlipidemia, hypertension, diabetes, smoking, etc.) increase the oxidative stress-reactive oxygen species(ROS) in the vessel wall by NADPH oxidase and xanthine oxidase, leading to dysequilibrium of $NO/O^{2-}$ and degrading NO bioavailability, and damaging endothelial function. SOD is a kind of anti-oxygen enzyme, being an anti-oxida-tive stress index to clear super-oxide anion. Quantifi-cation of active SOD can reflect the ability of clearing oxygen free radical. MDA, a kind of lipid per-oxida-tion metabolites, is an oxidative stress index and deter-mined active MDA can reflect impaired degree of cell by oxygen free radical. Both of them play important roles to keep $NO/O^{2-}$ balance. Quantification of serum SOD and MDA reflect oxidative stress taken place in the endothelium[8]. NO, one of vascular bioactive mol-ecules secreted by vascular endothelial cells, can relax smooth muscle cells of vascular wall, and suppress in-flammatory cells adhering to endothelial cell and inhibit oxygen free radical generation , which is an important protection mechanism for the pathogenesis of athero-sclerosis . Studies have shown that statins increased serum SOD, NO levels and decreased MDA level in plaque and endothelial tissue compared with non-simvastatin treatment[3,5,13-14]. However, rare data are now available demonstrating whether statins have therapeutic significance in the early stage of atherosclerosis in apolipoprotein E-deficient mice.

In the present study, there was no significant difference in serum cholesterol levels between treatment group and control group, but LDL levels were not determined in our study. Our results also showed that simvastatin significantly increased SOD activity and reduced MDA level independent of its cholesterol-lowering effect, which enhanced the anti-oxygen capability, lessened oxidative stress, and improved endothelial function. NO level were lower in both control group and treatment

group after 2 or 4 weeks simvastatin administration compared with that at the beginning of the study. But NO level in simvastatin group was much higher than that in control group at either 2 or 4 weeks, which suggested that simvastatin played an important role on revising endothelial dysfunction in apolipoprotein E-deficient mice . However, it had not been observed whether simvastatin could increase NO to the original level because the experimental time was short(2-4weeks). It is so pity that we can not determine the level of eNOS mRNA because of limited technology, which is the most direct mark for NO.

In summary, our results showed that simvastatin increased SOD activity, reduced MDA level, and inhibited NO lowering in the early stage of atherosclerosis in Apolipoprotein E-deficient mice, which were independent of its cholesterol-lowering effect, and suggested that simvastatin played important roles not only on second prevention but also on primary pre-vention of atherosclerosis. If simvastatin was used in earlier period of atherosclerosis, it might delay or even prevent atherosclerotic pathogenesis. In conclusion, therapeutic administration of statins at the early stage of atherosclerosis may revise endothelial dysfunction impaired due to any cardiovascular risk factor even when the subject's cholesterol level is "normal", which might be postulated to a new strategy for the primary prevention of atherosclerosis in the near future.

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