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*Research paper*

# Effects of Simvastatin on adiponectin and endothelial function in apolipoprotein E-deficient mice ☆

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## Abstract

**Objective:** To investigate the effects of simvastatin, a 3-hydroxy-3-methylglutaryl coenzyme A(HMG-CoA) reductase inhibitor, on adiponectin and markers of endothelial function in apolipoprotein E -deficient mice at an 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 4 weeks. Total cholesterol(TC), superoxide dismutase(SOD), malondialdehyde (MDA), and nitric oxide(NO) were measured by biochemical analysis, and adiponectin was measured by an ABC-ELISA method. **Results:** There was no significant difference in serum TC between control and treatment groups. Compared with the control animals, simvastatin-treated animals exhibited a significant increase in serum levels of adponectin, SOD and NO, and decrease in serum MDA($P < 0.01$). **Conclusion:** Simvastatin protects endothelial function by increasing serum adiponectin, which may increase serum SOD and NO, and decrease serum MDA. This study suggests that simvastatin has therapeutic advantages, unrelated to its cholesterol-lowering effect, that are mediated by adiponectin.

*Key words*: simvastatin; adiponectin; endothilial function; oxidative stress

## INTRODUCTION

There are many findings that clearly point toward statins having more than just a cholesterol lowering action. Previously, we demonstrated that statins directly increased NO and SOD levels, and decreased MDA levels in advanced atherosclerotic apolipoprotein E-deficient mice, and this effect of statins was associated with the inhibition of oxidative stress[1]. Adiponectin, an adipokine, has been shown to exert an array of remote endocrine effects through specific receptors expressed in the muscle, liver, and vasculature, resulting in an overall vasculoprotective effect, such that restoration of normal adiponectin secretion may have favorable cardiovascular effects[2-5]. Studies on the effects of statins on adiponcetin are rare. Thus, in the present study we have investigated the effects of simvastatin on apolipoprotein E-deficient mice and the relationships between adiponectin, oxidative stress and endothelial function.

## MATERIALS AND METHODS

This study was performed in accordance with the guidelines of the 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 and Use Committee.

### Animals

Male apoE-deficient mice on a C57BL/6J background, 6-weeks of age($n = 24$), were obtained from the Animal Center of Beijing University. Animals were randomly divided into two groups: control group (normal saline) and treatment group(simvastatin was

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obtained from Merck Inc., USA). 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 4 weeks. All animals were fed a standard chow ad libidum. The dose of simvastatin used in the study was based on that used in previous studies with hyperlipidemia mice[6,7].

### Animal Euthanasia and Serum Preparation

Blood was collected from the fossa orbitalis after 4 weeks simvastatin treatment, and then the animals were euthanized by guillotine after sedation(1% butaylone, 0.5-1.0 mL, which is an adequate dose of sedation).

### Determination of TC, SOD, MDA, and NO

Total cholesterol was determined using an automatic biochemistry analyzer(SABA218, Italian), and quality control(QC) serum was added; Serum SOD was measured by a xanthine oxidase method; MAD was determined by thiobarbituric acid method, $NO^{3-}$and $NO^{2-}$ were assessed using a nitrate reductase-Griessb reagent method, and the total amount of $NO^{3-}$and $NO^{2-}$ represented the NO levels. All kits were obtained from Nanjing Jiancheng Biotechnology Company(Nanjing, China).

### Measurement of adiponetin

Serum adiponectin was measured by a double antibody sandwich method ABC-ELISA method. Kits were obtained from Shanghai Xitang Biotechnology Company (Shanghai, China).

### Statistics analysis

All data are presented as mean±SEM values. Control and treatment group mean values of TC, SOD, MDA, NO and adiponectin were compared using 2-tailed Student *t* test, and $P \leqslant 0.05$ was considered significant. Linear correlations curves relating adiponectin and SOD, MDA and NO were generated using EXEL.

## RESULTS

### Effects of Simvastatin on Serum TC, SOD, MDA and NO Levels

After 4 weeks of simvastatin treatment there was no significant difference in the mean TC values of the control and treatment groups, whereas the statin treatment significantly decreased serum MDA levels($P < 0.01$), and increased both serum SOD levels($P < 0.01$) and NO levels($P < 0.01$). The simvastatin treatment also significantly increased serum adiponectin concentration compared to control values($P < 0.01$)(***Table 1***).

***Table 1*** **Effects of Simvastatin on Serum TC, SOD, MDA and NO Levels**

| Group($n$=12) | TC(mmol/l) | Adiponectin(mg/ml) | SOD(u/l) | MDA(nmol/l) | NO(umol/l) |
|---|---|---|---|---|---|
| Control | 10.8014 ± 2.0984 | 1.0004 ± 0.2202 | 97.2866 ± 7.6414 | 17.3846 ± 1.0562 | 12.3656 ± 2.1816 |
| Simvastatin | 11.8963 ± 0.2036$^{NS}$ | 1.4960 ± 0.2480$^{\#}$ | 135.3897 ± 5.5664$^{\#}$ | 10.5641 ± 0.5941$^{\#}$ | 28.4946 ± 4.1529$^{\#}$ |

Compared with control group, NS, $P > 0.05$; $^{\#}P < 0.01$.

Data from control animals and animals treated with simvastatin for 4 weeks were used in linear regression plots of serum adiponectin levels versus serum NO, SOD and MDA levels(***Fig. 1~3***). Both NO and SOD showed a positive correlation with adiponectin levels, while MDA showed a negative correlation. The $r^2$ values were all above 0.6, and the correlations were significant ($P < 0.01$).

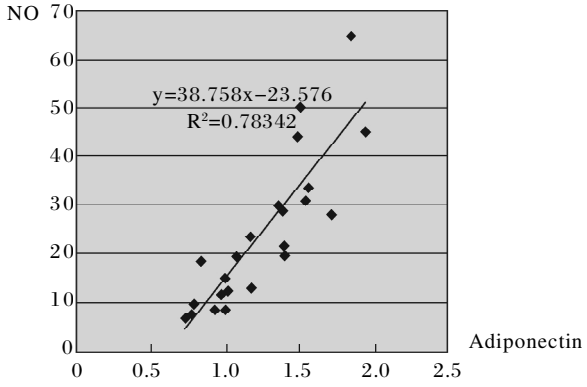


***Fig. 1*** Correlation between adiponectin and NO($P < 0.01$)

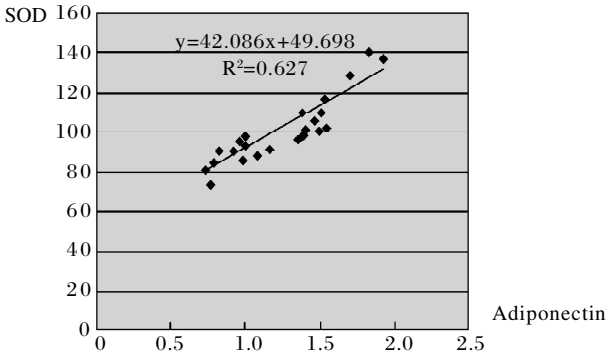


***Fig. 2*** Correlation between adiponectin and SOD($P < 0.01$)

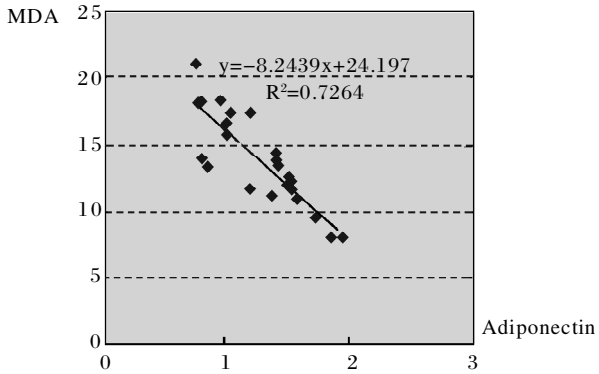


***Fig. 3*** Correlation between adiponectin and MDA($P < 0.01$)

## DISCUSSION

Adiponectin, referred to as 30-kDa adipocyte complement-related protein or 28-kDa gelatin-binding protein, consists of a carboxyl-terminal globular domain, a variable region, and an aminoterminal collagen-like domain containing 22 Gly-X-Y repeats[8]. Most adipokines are proinflammatory. In contrast, adiponectin, is an antiinflammatory adipokine that is abundantly present in blood. Whereas adiponectin is expressed almost exclusively in adipose tissue[8,9], plasma adiponectin levels are paradoxically decreased in obese individuals[10]. Low plasma adiponectin levels, also known as hypoadiponectinemia, are closely associated with the increased prevalence of coronary heart disease, and hypertension[4]. Studies have also shown that hypoadiponectinemia is associated with elevation of circulating C-reactive protein(CRP) levels, and a significant negative relationship is found between CRP and adiponectin mRNA levels in adipose tissues[11,12].

The observation of Xu and colleagues suggests that adiponectin exerts a common protective effect in the endothelium[13]. Several adiponectin cardiovascular protective mechanisms have been identified. Adiponectin inhibits NF-[kappa]B activation in endothelial cells, at least in part due to its ability to activate AMPK. Inhibition of NF-[kappa]B by adiponectin results in downregulation of CRP, IL-8, and adhesion molecule expression. In macrophages, adiponectin attenuates TNF-[alpha] production through its ability to suppress NF-[kappa]B activation, and it promotes the clearance of apoptotic cells by macrophages[14,15]. Adiponectin causes beneficial responses in endothelial cells that oppose diverse adverse influences, including the effects of Ang Ⅱ and TNF-[alpha], which are known to trigger different cellular signaling pathways[13,15]. Adiponectin promotes differentiation of endothelial cells from progenitor-circulating monocytes, thereby providing the opportunity for intimal healing through improved arterial endothelialization[12].

Cardiovascular risk-factors, such as smoking, hypertention, diabetes and hyperlipidemia increase the oxidative stress-reactive oxygen species(ROS) in the vessel wall by nicotinamide adenine dinucleotide phosphate(NADPH) oxidase and xanthine oxidase, leading to dysequilibrium of $NO/O^{2-}$ and the degradation of NO bioavailability, and damaging endothelial function. The apolipoprotein E-deficient mouse is an excellent atherosclerosis animal model with a natural history and plaque distribution similar to that of humans[7]. Studies showed that monocytes attach to impaired apolipoprotein E-deficient mouse endothelial cells at 6 weeks of age, long before fibrous plaque formation. Thus, endothelial dysfunction had already begun. Quantification of active SOD can reflect the ability of tissue to clear oxygen free radicals. MDA, a lipid peroxidation metabolite, and is an oxidative stress index. Determining MDA can reflect the degree of cell impairment by oxygen free radicals. Both SOD and MDA play important roles in maintaining the $NO/O^{2-}$ balance[6]. NO, a biomarker of endothelial function, can relax vascular smooth muscle, and suppress inflammatory cells adhering to endothelial cell, and inhibit oxygen free radical generation, thereby acting as an important protective mechanism against the pathogenesis of atherosclerosis[6].

In the present study we have demonstrated that with 4 weeks of statin therapy there was no significant difference in serum cholesterol levels, there was a significant increase in adiponectin levels, while SOD activity and NO levels increased, and MDA levels were reduced. Further, adiponectin showed a positive correlation with NO and SOD, and a negative correlation with MDA. This positive correlation between adiponectin and NO suggests that statins have a therapeutic function in apolipoprotein E-deficient mice through the restoration of normal circulating adiponectin. Studies have also shown that adiponectin increases NO production in endothelial cells[16]. There are two mechanisms to explain adiponectin's increase in NO production: first, as previously mentioned, adiponectin has a general protective role in endothelial cells and would thereby be expected to maintain NO synthesis; second, adiponectin opposes proatherosclereteic effects, such as that of Ang Ⅱ which stimulates NADPH oxidase, so adiponectin helps to maintain the equilibrium of $NO/O^{2-}$ and increases NO bioavailability. In these ways, the reduction of oxidative stress by adiponetin would be expected to increase SOD levels and decrease MDA. These mechanisms are worth further exploration. It should be mentioned, however, that serum adiponectin levels in simvastatin-treated apolipoprotein E-deficient mice were still quite low compared to wild type mice($1.50 \pm 0.25$ μg/ml vs approximately 10 μg/ml).

In summary, the present study provides evidence that simvastatin treatment exerts important cholesterol-independent pleiotropic effects in $apoE^{-/-}$ mice which are mediated by a partial restoration of serum adiponetin, which appears to improve endothelial function. This study also supports the theory that in the early stage of artherosclerosis, statin treatment exerts effects that are independent of a cholesterol-lowering effect. In more general terms, the data presented here suggest that those beneficial effects of simvastatin that are cholesterol-independent make it ideal for use in patients who are at risk for atherosclerosis, but who do

not yet have plaque formation. These types of observation provide additional explanation for the benefits of statin therapy in diabetic patients and patients with cardiovascular disease who have normal or low LDL.

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