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Protective effects of lovastatin on vascular endothelium injured by low density lipoprotein

MA Feng-Xia, LIU Li-Ying¹, XIONG Xiao-Ming

Department of Pharmacology, Pharmaceutical College, Central South University, Changsha 410078, China

KEY WORDS lovastatin; lipoproteins; endothelium; muscle relaxation; nitric oxide; malondialdehyde; superoxide dismutase; rats

ABSTRACT

AIM: To examine protective effects of lovastatin, an inhibitor of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase, on endothelial dysfunction induced by a single intravenous injection of natural low density lipoprotein (n-LDL) and analyze the possible action mechanism of lovastatin. **METHODS:** Rats were treated by intraperitoneal injection with lovastatin at dose of 2 or 4 mg/kg body weight once daily for 7 d, and on d 6 a single injection of n-LDL 4 mg/kg was given by sublingual vein. Forty eight hours after injection of n-LDL, the descending thoracic aorta of rats was taken. Acetylcholine (ACh)-induced endothelium-dependent relaxation (EDR) and sodium nitroprusside (SNP)-induced endothelium-independent relaxation of aortic rings were examined *in vitro*. Malondialdehyde (MDA), nitric oxide (NO), and activity of superoxide dismutase (SOD) as well as level of lipid in serum were analyzed. **RESULTS:** A single injection of n-LDL inhibited ACh-induced EDR compared with normal control group (maximal relaxation rate: 69.5 %±1.2 % vs 91.0 %±1.2 %, $P<0.05$), decreased serum NO level [(7.0±0.5) μmol/L vs (11.2±0.9) μmol/L, $P<0.05$] and serum SOD activity [(371±16) kNU/L vs (405±18) kNU/L, $P<0.05$] and elevated serum MDA level [(5.4±0.5) μmol/L vs (3.0±0.8) μmol/L, $P<0.05$]. Compared with n-LDL treated group, lovastatin 2 and 4 mg/kg increased EDR (maximal relaxation rate 82.9 %±0.5% and 83.7 %±0.7 % vs 69.5 %±1.2 %, $P<0.05$) and elevated NO level [(11.0±0.7) and (11.2±0.8) μmol/L vs (7.0±0.5) μmol/L, $P<0.05$], increased SOD activity [(402±15) and (408±25) kNU/L vs (371±16) kNU/L, $P<0.05$], and reduced serum MDA level [(3.3±0.6) and (3.5±0.4) μmol/L vs (5.4±0.5) μmol/L, $P<0.05$]. But sodium nitroprusside-induced endothelium-independent relaxation and the level of serum lipid in both saline+LDL group and lovastatin-treated group had no marked alteration. **CONCLUSION:** Lovastatin was able to protect vascular endothelium from dysfunction induced by a single injection of n-LDL.

INTRODUCTION

Accumulation of low density lipoprotein (LDL) in the arterial wall appears to play a crucial role in the

initiation and progression of atherosclerotic plaque. Lovastatin is used widely for the treatment of hypercholesterolemia, the mechanism of its effect of reducing serum cholesterol involves competitively inhibiting 3-hydroxy-3-methylglutaryl (HMG)-CoA reductase. Recently large clinical trials have demonstrated that HMG-CoA reductase inhibitors (statins) decreased the incidence of ischemic strokes and myocardial infarctions

¹ Correspondence to Prof LIU Li-Ying.

Phn 86-731-480-5441. E-mail xjjz@cs.hn.cn

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in atherosclerotic and hypercholesterolemic individuals. Although the beneficial effects of statins are primarily attributed to their lipid-lowering effects, the analyses of the data from these trials suggest that there may be beneficial effects of these agents that are independent of serum cholesterol level^[1]. Beyond their effects on serum cholesterol levels, non-lowering-cholesterol effects of statins involve improving or restoring endothelial function, decreasing oxidative stress and vascular inflammation as well as enhancing the stability of atherosclerotic plaques^[2]. It has been known that high LDL is a key factor of atherogenesis. It has been well demonstrated that presence of LDL and oxidatively modified LDL (Ox-LDL) in atherosclerotic lesion as well as Ox-LDL-induced inhibition of the endothelium-dependent relaxation of arteries may be related to cardiovascular disease induced by atherosclerosis^[3,4]. Recent data suggests that statins ameliorate endothelial function^[5]. However these data were from cellular culture, atherosclerotic patients, or animal models of hyperlipidemia. It was reported that a single injection of n-LDL induced accumulation of LDL in the arterial wall and formation of membrane-like whorls inside the internal elastic membrane^[6]. However, to our knowledge, no study so far has demonstrated that statins are able to prevent vascular endothelium from a direct injury induced by n-LDL. To demonstrate this point, we observed effects of lovastatin on endothelial dysfunction of arteries and changes of biochemical indexes induced by a direct intravenous injection of n-LDL.

MATERIALS AND METHODS

Drugs and chemicals Lovastatin was kindly presented by Merk, Sharp & Dohme Inc and dissolved in solvent composed of ethanol and propylene glycol (2 g/L lovastatin was prepared) before injection. Sodium nitroprusside (SNP), acetylcholine (ACh), phenylephrine (Phe), *N*^o-nitro-*L*-arginine (*L*-NNA) were purchased from Sigma Chemical Co, USA. The kit for nitric oxide (NO), malondialdehyde (MDA), and superoxide dismutase (SOD) assay were purchased from Nanjing Jiancheng Biological Institute.

Animals Male Sprague-Dawley rats were supplied by the Animal Center of Xiangya Medical College of Central South University. They were fed with standard chow and given free access to water until they were killed. Their weight at the start of the experiment was (180±20) g.

Preparation of LDL LDL was isolated by discontinuous density gradient ultracentrifugation method from fresh human plasma in healthy normal-lipidemic donors as previously described in our experiment^[7]. Edetic acid 10 µmol/L was added to the plasma to avoid autoxidation and its density was adjusted to 1.065 g/L by adding solid NaBr and the samples were ultracentrifugated (200 000×g, 20 h, 4 °C). The purity of LDL was identified by agarose gel electrophoresis and the protein content was determined by the method of Lowry *et al*. The purified LDL was dialyzed for 24 h against 2 L phosphate buffer solution (pH 7.4, 0.2 mol/L) at 4 °C. Dialyzed LDL was frozen at -70 °C. Before the injection of n-LDL into the animals, the LDL was sterilized after passing through a 0.45-µm filter and was kept on ice until injected.

Protocol of experiment Forty eight healthy male Sprague-Dawley rats were divided randomly to 6 groups with 8 rats in each group. (1) Normal control group: 8 rats were ip injected with saline at dose of 2 mL/kg once daily for 7 d. (2) Saline+LDL group: 8 rats were ip injected with saline at dose of 2 mL/kg once daily for 7 d, and on d 6 rats were given a single injection of n-LDL (4 mg/kg body weight) by the sublingual vein under ether anesthesia. (3) Solvent+ LDL group: 8 rats were ip injected with solvent composed of ethanol and propylene glycol (propylene glycol:ethanol=9:1) at dose of 2 mL/kg once daily for 7 d, and on d 6 rats were given a single injection of n-LDL as saline+LDL group. (4) Lovastatin-treated group: 16 rats were injected ip lovastatin at dose of 2 or 4 mg/kg body weight once daily for 7 d^[8], and on d 6 rats were given a single injection of n-LDL (5) *L*-NNA+ lovastatin-treated group: 8 rats were injected ip lovastatin 4 mg/kg body weight once daily for 7 d and *L*-NNA 20 mg/kg daily for 3 d in the first three days of lovastatin injection, and on d 6 rats were given a single injection of n-LDL.

All rats were fasted and received last injection of lovastatin or saline or solvent 24 h before sacrifice, 48 h after rats were given a single injection of n-LDL. Blood samples were drawn from carotid artery and thoracic aorta were taken under anesthesia.

Aortic rings preparation and bioassay of vasoreactivity Rats were killed by exsanguination after anesthesia with pentobarbital sodium (30 mg/kg, ip). The descending thoracic aorta was dissected and aortic rings approximately 4-mm in length were prepared. For isometric force recording, aortic ring was mounted between two stainless steel hooks and suspended in a

6-mL organ bath containing 5 mL Krebs' solution composed of (mmol/L): NaCl 118; KCl 4.8; CaCl₂ 2.5; MgSO₄ 1.2; NaH₂PO₄ 1.2; NaHCO₃ 24; glucose 11; and edetic acid 0.03; at 37 °C bubbled with 95 % O₂+5 % CO₂ gas mixture. The tension of ring was monitored by a force transducer and recorded on a polygraph (Modle YL-I, Chengdu Instruments, China). An initially load of 2 g on resting tension was applied. After 90 min equilibration period, the ring was precontracted by Phe 0.1 μmol/L. When the developed tension attained its peak value, the rings were relaxed by ACh (0.003-3 μmol/L) or SNP (0.001-0.3 μmol/L) and concentration-response curves induced by ACh or SNP were recorded. The ACh-induced endothelial dependent relaxation and SNP-induced endothelial independent relaxation were calculated as percentage of the contraction to Phe.

Assay of serum total nitrate and nitrite (NO₂ +NO₃⁻) Forty eight hours after rats were injected n-LDL, 5 mL of blood was drawn from carotid artery and stored in refrigerator for 1 h, then blood was centrifugated (3000×g) for 15 min at 4 °C to collect serum. NO was assayed by Griess method. The absorption was determined at 540 nm with a spectrophotometer^[9].

Determination of serum MDA content Serum MDA was measured by the thiobarbituric acid reactive substances (TBARS) assay^[10]. Briefly, 20 % trichloroacetic acid 1.0 mL was added to 100 μL serum, then 1 % TBARS reagent (1.0 mL) was added, mixed, and incubated at 100 °C for 30 min. After cooling on ice, samples were centrifugated at 1000×g for 20 min and absorbance of the supernatant was read at 532 nm. Blanks for each sample were prepared and assessed in the same way to correct for the contribution of A₅₃₂ to the sample. TBARS results were expressed as MDA equivalents using tetraethoxypropane as standard.

Determination of serum total superoxide dismutase activity^[11] Serum 0.5 mL with 50 μmol/L xanthine and 2.5 μmol/L xanthine oxidase in 50 mmol/L potassium phosphate buffer (pH 7.8, 37 °C) were incubated for 40 min and NBT was added. Then blue formazan was monitored spectrophotometrically at 550 nm. The amount of protein that inhibits NBT reduction to 50 % of maximum is defined as 1 nitrite unit (NU) of SOD activity.

Statistical analysis All the data were expressed as mean±SD and analyzed by one way ANOVA and Newman-Keuls method.

RESULTS

Endothelium-dependent relaxation Forty eight hours after LDL injection, the maximal relaxation of aortic ring induced by ACh was decreased compared with normal control (69.5 %±1.2 % vs 91.0 %±1.2 %, *n*=8, *P*<0.01). The maximal relaxation rate in lovastatin 2 and 4 mg/kg-treated group was 82.9 %±0.5 % or 83.7 %±0.7 % (*n*=8), respectively (Fig 1). *L*-NNA inhibited the protective effect of lovastatin against n-LDL. The maximal relaxation rate was 75.2 %±1.1 % (*n*=8, *P*<0.01, Fig 2). The solvent of lovastatin had no

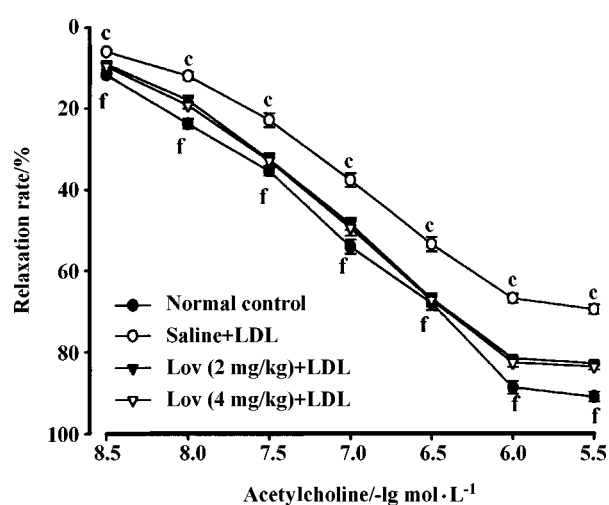


Fig 1. Dose-response curves of ACh-induced endothelium-dependent relaxation of rat aortic rings in different groups. *n*=8. Mean±SD. ^c*P*<0.01 vs normal control group. ^f*P*<0.01 vs saline+LDL group.

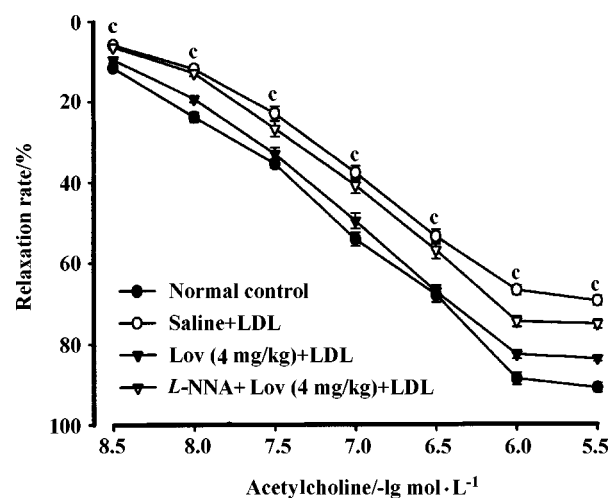


Fig 2. Effect of *L*-NNA on ACh-induced endothelium-dependent relaxation of rat aortic rings. *n*=8. Mean±SD. ^c*P*<0.01 vs Lov 4 mg/kg+LDL group.

effect on EDR of aortic rings (Fig 3).

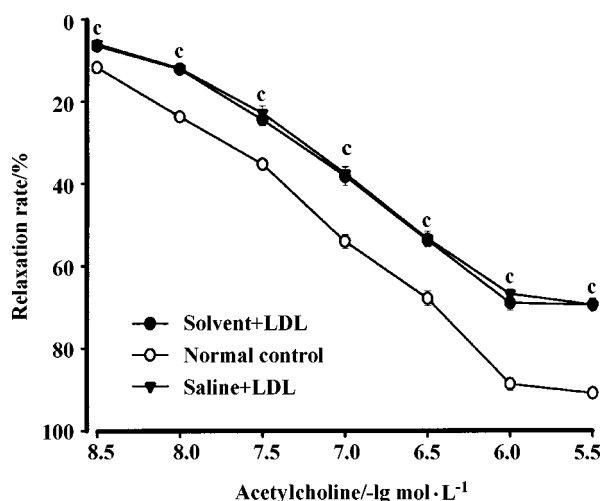


Fig 3. Effect of solvent on ACh-induced endothelium-dependent relaxation of rat aortic rings. *n*=8. Mean±SD. ^c*P*<0.01 vs normal control group.

Endothelium-independent relaxation of aortic rings A single injection of LDL has no remarkable effect on SNP-induced endothelium-independent relaxation response of aortic rings. The maximal relaxation rate of aortic ring was 95.2 %±2.1%, 94.6 %±1.8 %, and 95.6 %±1.3 % (*n*=8) in normal control group, saline+LDL group, and lovastatin+LDL group, respectively. The data between three groups had no significant difference.

Serum NO level Forty eight hours after n-LDL injection, serum NO level was significantly lower in saline+LDL group than that of normal control group [(8.0±0.5) vs (12.2±0.9) μmol/L, *P*<0.01], in agreement with the decrease of relaxation rate of aortic rings. Lovastatin 2 and 4 mg/kg remarkably elevated NO level [(11.0±0.7) μmol/L and (11.2±0.8) μmol/L, *n*=8, vs saline+LDL group, *P*<0.01]. *L*-NNA reduced the protective effect of lovastatin. NO level was (7.3±1.3) μmol/L (*n*=8, *P*<0.01 vs Lov 2 or 4 mg/kg). The solvent had no effect on serum NO level.

Serum SOD activity A single injection of n-LDL decreased serum SOD [(371±16) kNU/L vs (405±18) kNU/L of normal control, *n*=8, *P*<0.05]. Lovastatin 2 and 4 mg/kg increased SOD activity [(402±15) kNU/L and (408±25) kNU/L, *n*=8, *P*<0.05 vs saline+LDL group]. *L*-NNA reduced the beneficial effect of lovastatin, the SOD level was (381±15) kNU/L (*n*=8, *P*<0.05 vs lovastatin-treated group).

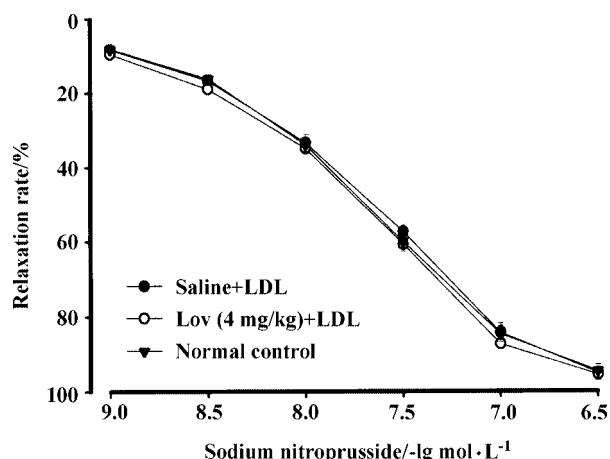


Fig 4. Dose-response curve of SNP-induced endothelium-independent relaxation of rat aortic rings in different treated groups. *n*=8. Mean±SD.

Serum MDA level A single injection of n-LDL remarkably increased serum MDA level [(5.9±0.8) μmol/L vs (3.0±0.8) μmol/L, *n*=8, *P*<0.01 vs normal control]. Lovastatin 2 and 4 mg/kg inhibited the increase of serum MDA level [(3.3±0.6) and (3.5±0.4) μmol/L vs (5.9±0.8) μmol/L, *n*=8, *P*<0.01]. *L*-NNA reversed the inhibitory effects of lovastatin. MDA level was (5.6±0.6) μmol/L (*n*=8). Solvent had no effect on serum NO level.

Serum lipid level A single injection of n-LDL failed to affect serum lipid level. Treatment with lovastatin 4 mg/kg for 7 d, the lipid level had no remarkable alteration (Tab 1).

Tab 1. Serum lipid level in different treated groups. *n*=6. Mean±SD. ^a*P*>0.05 vs normal control group. ^d*P*>0.05 vs saline+LDL group.

Groups	TC/mg·L ⁻¹	TG/mg·L ⁻¹	HDLC/mg·L ⁻¹	LDLC/mg·L ⁻¹
Normal control	535±38	624±102	656±82	123±22
Saline+LDL	588±62 ^a	643±78 ^a	610±90 ^a	151±13 ^a
Lov 4 mg/kg+LDL	555±66 ^d	704±59 ^d	680±31 ^d	149±17 ^d

DISCUSSION

In the present study, we used a new simple animal model in which a single injection of unmodified, heterologous LDL was given in the sublingual vein of rats and observed protective effects of lovastatin against

n-LDL-induced endothelial dysfunctions. The results showed that ACh-induced endothelial dependent relaxation (EDR) of aortic rings in rats with a single injection of n-LDL was significantly inhibited, simultaneously associated with reduction of both NO level and SOD activity as well as increase of MDA concentration in serum. After treatment with lovastatin 2 or 4 mg/kg once daily for 7 d, inhibition of ACh-induced EDR and changes of above biochemical indexes induced by n-LDL were significantly ameliorated.

Calara F *et al*^[6] reported that a single injection of unmodified LDL induced accumulation of LDL in the arterial wall which peaked at 12 h, moreover, LDL was oxidatively modified within 6 h and peaked at 24 h. The electron microscopic observation showed that 24 h after injection of LDL, local collection of lipid bilayers was apparent and membrane-like whorls were formed inside the internal elastic membrane. It was supposed that circulating LDL was taken up locally in large amounts in the arterial wall *via* the endothelium and then penetrated into the media to generate free lipid molecules. In addition to Ox-LDL-induced activation of nuclear factor kappa B (NF- κ B), expression of intercellular adhesion molecule-1 (ICAM-1) in protein level as well as mRNA level in vascular endothelium also has been observed. Thus it is documented that this model is able to be used to evaluate rapidly the effects of therapeutic drugs on the early events in atherogenesis. Jiang *et al*^[12] used this model to induce endothelial dysfunction of rat aortic rings and found that endothelial injury peaked at 48 h after a single intravenous injection of n-LDL at dose of 4 mg/kg. The advantage of the present model is much less time consumed (only 1-2 d in contrast with a few months in models induced by atherogenic diet), simpler in procedure, but similar in principal properties as early atherosclerosis produced by atherogenic diet.

Our study found that ACh-induced EDR was maximally inhibited at 48 h after injection of n-LDL and associated with reduction of NO level in serum. However, SNP-induced endothelium-independent relaxation was not inhibited. An important characteristic of endothelial dysfunction is the impaired synthesis, release, and activation of endothelial-derived nitric oxide. Reactive oxygen species generated by a variety of cells existing in the atherosclerotic vessels or Ox-LDL are profoundly implicated in the pathogenesis of atherosclerosis by promoting macrophage foam cell formation and inducing

endothelial cell damage. Superoxide anion is one of the strongest oxidants and plays a central role as the source of many reactive oxygen species. The SOD is an important antioxidative enzyme to scavenge superoxide anion which is lowered in the process of atherogenesis. We supposed that inhibition of EDR induced by n-LDL may also involve superoxide anion produced by interaction of LDL or Ox-LDL with vascular endothelial cell as well as various blood cell. Lipid peroxidation are formed when reactive oxygen species react with an unsaturated fatty acid chain in membrane lipids. MDA is one of low-molecular-weight end products formed via the decomposition of certain primary and secondary lipid peroxidation products^[13]. SOD activity was lowered and concentration of MDA was elevated, which was able to interpret that inhibition of EDR induced by a single injection of n-LDL involved reactive oxygen species and lipid peroxidation *in vivo*.

The present study directly documented protective effects of lovastatin against endothelial dysfunction induced by n-LDL *in vivo*. Lovastatin as an important cholesterol-lowering drug, by inhibiting HMG-CoA reductase and inhibiting *L*-mevalonic acid synthesis, an intermediate of the cholesterol biosynthetic pathway, finally reduces cholesterol biosynthesis. Recently, it was reported that statins were able to reverse endothelial dysfunction induced by atherosclerosis and this beneficial effect of statins derived partially from direct actions on the endothelial vasoactive factors, nitric oxide and endothelin-1^[14]. In our study, lovastatin was given only for 7 d and the serum lipid level had no remarkable change. However, Our study results showed that lovastatin protected endothelium, increased NO level and SOD activity, and reduced MDA concentration. So the beneficial effects of lovastatin were contributed to its non-lipid-lowering effects. Furthermore, the protective effect of lovastatin was reduced by pretreatment with *N*^o-nitro-*L*-arginine (*L*-NNA), a non-selective nitric oxide synthase (NOS) inhibitor and lovastatin had no effect on SNP-induced endothelium-independent relaxation. These results provided a new potent direct evidence for statins. Some statins were able to inhibit production of proinflammatory cytokines that regulated many key functions of the vascular wall including monocyte adhesion, chemotaxis, and metalloproteinase secretion^[15], so the mechanism of protective effects of lovastatin on endothelium may be related to above described action of statin, but further study is needed.

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