Aspirin protected against endothelial damage induced by LDL: role of endogenous NO synthase inhibitors in rats[1]

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ABSTRACT

AIM: To study the protective effect of aspirin on damages of the endothelium induced by low-density lipoprotein (LDL), and whether the protective effect of aspirin is related to reduction of nitric oxide synthase inhibitor level. **METHODS:** Vascular endothelial injury was induced by a single injection of native LDL (4 mg/kg) in rats. Vasodilator responses to acetylcholine (ACh) in the isolated aortic rings were determined, and serum concentrations of asymmetric dimethylarginine (ADMA), malondialdehyde (MDA), tumour necrosis factor-α (TNF-α), and the activity of dimethylaminohydrolase (DDAH) were measured. **RESULTS:** A single injection of LDL (4 mg/kg) significantly decreased vasodilator responses to ACh, increased the serum level of ADMA, MDA, and TNF-α, and decreased DDAH activity. Aspirin (30 or 100 mg/kg) markedly reduced the inhibition of vasodilator responses to ACh by LDL, and the protective effect of aspirin at the lower dose was greater compared with high-dose aspirin group. Aspirin inhibited the increased level of MDA and TNF-α induced by LDL. Aspirin at the dose of 30 mg/kg, but not at higher dose (100 mg/kg), significantly reduced the concentration of ADMA and increased the activity of DDAH. **CONCLUSION:** Aspirin at the lower dose (30 mg/kg) protects the endothelium against damages elicited by LDL *in vivo*, and the protective effect of aspirin on endothelium is related to reduction of ADMA concentration by increasing DDAH activity.

INTRODUCTION

A great deal of information has been demonstrated that endothelial function is impaired in humans and animals with hypercholesterolemia. It has been suggested that endothelium-derived nitric oxide (NO), which mediates endothelial-dependent relaxation, is synthesized from *L*-arginine by NO synthase (NOS) in endothelial cells. Recently, it has been found that endogenous inhibitors of NOS such as asymmetric dimethylarginine (ADMA), which inhibits nitric oxide synthesis, are significantly increased in animals and patients with chronic hypercholesterolemia[1,2], and ADMA has been thought to be a key factor contributing endothelial dysfunction.

The substantive accumulation of low-density lipoprotein (LDL) in the arterial wall where it becomes oxidatively modified LDL (ox-LDL), which impairs en-

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dothelial function in the hypercholesterolemic animal and man, has been suggested to be a key event in early atherosclerosis[3]. Previous observations have shown that a single injection of native LDL caused a rapid accumulation and oxidation of LDL in the arterial wall[4,5]. In cultured endothelial cells, incubation with LDL caused histomorphological and functional damages[6]. Our recent work found that LDL caused endothelial dysfunction concomitantly with an elevation of ADMA level[4,7]. In cultured endothelial cells, ox-LDL induced a marked increase in the level of ADMA[8].

Aspirin, the most widely prescribed nonsteroidal anti-inflammatory drug, is also one of potent antioxidant compounds known[9]. A great deal of clinical studies have proved that aspirin at the lower dose reduces cardiovascular risk[10]. Aspirin has been shown to improve the endothelium-dependent relaxation of hypercholesterolemic animals[11]. However, the mechanisms responsible for the beneficial effect of aspirin on endothelial cells remain unclear. According to facilitation of the elevation of ADMA by lipid peroxide[12] and antioxidant properties of aspirin, in the present study we examined whether the protective effect of aspirin on endothelial dysfunction by intravenous injection of unmodified human LDL was related to reduction of ADMA level in rats.

MATERIALS AND METHODS

Reagents Aspirin, ADMA, 1,1-diphenyl-2-picrylhydracyl (DPPH), phenylephrine, and acetylcholine were purchased from Sigma. Vitamin E was obtained from Xiamen Fish Liver Oil Factory (Xiamen, China). Malondialdehyde (MDA) and tumor necrosis factor-α (TNF-α) assay kits were from Ju-Li Biological Medical Engineer Institute (Nanjing, China) and Immunity Institute of Dongya (Beijing, China), respectively. Intercellular Adhesion Molecule-1 (ICAM-1) immunohistochemistry kit was from Boster Biotechnology Co Ltd.

Animal model Male Sprague-Dawley rats weighing 180-220 g were obtained from the Animal Center of Xiang-Ya School of Medicine (Grade II, Certificate No 20-010). Vascular endothelial injury was induced by a single injection of native LDL (4 mg/kg, iv). The rats were pretreated with aspirin (30 or 100 mg/kg) or vitamin E (100 mg/kg) once a day for 5 d and then treated with LDL (4 mg/kg, 48 h). Aspirin and vitamin E were given orally.

LDL isolation Native LDL was isolated from freshly prepared normal human plasma through sequential density gradient ultracentrifugation in sodium bromide density solutions in the density range 1.019-1.063 kg/L as previously described[13]. Then LDL was dialyzed against 0.01 mol/L PBS (pH 7.4) containing 0.01 % EDTA and stored at 4 ºC in the dark. Protein concentration was measured by Lowry's method.

Organ chamber experiments The rats were anesthetized with sodium pentobarbital (60 mg/kg, ip). After blood samples were collected from the carotid artery, the thoracic aorta was rapidly isolated and cut into rings of 3-mm in length. The rings were suspended horizontally between two stainless steel wires and mounted in a 5-mL organ chamber filled with warmed (37 ºC) and oxygenated (95 % O_2 and 5 % CO_2) Krebs' solution. The Krebs' solution had the following composition (mmol/L): NaCl, 119.0; $NaHCO_3$, 25.0; KCl, 4.7; KH_2PO_4, 1.2; $MgSO_4 \cdot 7H_2O$, 1.2; $CaCl_2$, 2.5; and glucose, 11.0. One end of ring was connected to a force transducer. The aortic ring was stretched with 2 g resting force and equilibrated for 60 min, and then precontracted with KCl (60 mmol/L). After a maximal response to KCl was obtained, the rings were washed repeatedly with Krebs' solution and equilibrated again for 30 min. In order to measure relaxation responses, rings were contracted with phenylephrine to 40 %-50 % of their maximal contraction. After the contraction stablized, an accumulative concentration-response curve to ACh (3×10^{-9}-1×10^{-6} mol/L) was observed[7].

Determination of ADMA concentration The blood samples were centrifugated at 1300×*g* for 15 min (4 ºC) and the serum was deproteined with 5-sulfosalicylic acid (5-SSA). The supernatant was measured by high-performance liquid chromatography (HPLC) as described previously with some modification. HPLC was carried out using a Shimadzu LC-6A liquid chromatograph with Shimadzu SCL-6A system controller and Shimadzu SIC-6A autosampler. *o*-Phthaldialdehyde adducts of methylated amino acids and internal standard ADMA produced by precolumn mixing were monitored using a model RF 530 fluorescence detector set at λ_{ex}=338 and λ_{em}=425 nm on a Resolve C_{18} column. Samples were eluted from the column using a linear gradient containing mobile phase A composed of 0.05 mol/L (pH 6.8) sodium acetate-methanol-tetrahydrofuran (81:18:1 v:v:v) and mobile phase B composed of 50 μmol/L sodium acetate-methanol-tetrahydrofuran (22:77:1 v:v:v) at a flow-rate of 1 mL/min[14].

DDAH activity assay The activity of DDAH was

estimated by measuring *L*-citrulline formation in red blood cell lysates, as describe previously. In an ice bath, cell lysates were divided into 2 groups, and ADMA was added (final concentration 0.4 mmol/L). To inactivate DDAH, 10 % trichloroacetic acid was immediately added to one experimental group. This group provided a baseline of 0 % DDAH activity. The other lysate was incubated at 37 ºC for 4 min before the addition of 10 % trichloroacetic acid. The amounts of *L*-citrulline formed were measured by the method of Precott and Jones. The difference in *L*-citrulline concentrations between two groups reflected the DDAH activity. For every experiment, DDAH activity of red blood cells in control group was defined as 100 %, and DDAH activity in other group was expressed as percentage of *L*-citrulline from ADMA compared with control[15].

Determination of MDA and TNF-α concentration in serum The content of thiobarbituric acid reactive substance reflecting levels of lipid peroxide was measured spectrophotometrically, by described methods of assay kits and expressed as the amount of MDA.

Blood sample was collected in tubes. Serum was obtained by centrifugation at 1300×*g* for 15 min (4 ºC). The content of TNF-α in serum was determined with a commercially radioimmunoassay kit, using antisera raised against rat TNF-α, ^{121}I-labelled TNF-α, and TNF-α standard.

Determination of ICAM-1 content in the aorta After collection of blood sample, the thoracic aorta was rapidly removed and fixed in 4 % paraformaldehyde in 0.1 mol/L phosphate-buffer saline (PBS) for 10 h and followed by cruopretection with 30 % sucrose in PBS for 12 h. Aorta sections, 20 μm in thickness, were cut transversely in a cryostat (AO, USA) at -20 ºC.

After a preliminary wash in PBS and treatment with 0.3 % H_2O_2 to quench endogenous peroxidase activity, free-floating sections were washed in PBS containing 1 % Triton X-100 and 1 % normal goat serum albumin for 2 h. Then, sections were incubated with the primary antibody against-rat ICAM-1 (rabbit polyclonal antibody) at a dilution of 1:1000 for 12 h at 4 ºC. Sections were incubated with biotinylated secondary antibody diluted to 1:100 for 30 min, and then with streptavidin-biotin-enzyme complex (SABC) diluted to 1:100 for 30 min at room temperature. After each incubation sections were washed three times with PBS for a total of 15 min. The diaminobenzidine (0.02 %) and H_2O_2 (0.01 %) were employed for color reaction to defect positive signal. Finally, sections were mounted on slides, dehydrated through an alcohol range, cleared in xylene, and coverslipped. Slides were viewed under a microscope (Olympus, Japan) and photographed by a digital camera (Nikon, Japan) coupled to computer. The content of ICAM-1 in the thoracic aorta was measured by the immunostaining area using a High Resolution Color Image Analysis System (Tongji Medical University, Wuhan, China). We took readings from 5 to 6 sections from each of five sections to be averaged for each animal.

Effect of aspirin on DPPH radicals The 2,2-diphenyl-1-picryl-hydrazyl (DPPH), a stable free radical, was used to determine free radical scavenging capacity of aspirin. A 3-mL DPPH in ethanol solution (50 μmol/L) was mixed with 50 μL different concentrations (3×10^{-5}-1×10^{-2} mol/L) of aspirin or vitamin E in a cuvette, and the absorbance change at 517 nm was measured 5 min later with a spectrophotometer. At the same time, a blank solution of DPPH was measured to estimate DPPH decomposition during the time of measurement. Measurements were performed at least in triplicate. Inhibition of coloration was expressed as a percentage and EC_{50} value was obtained from the inhibition curve.

Statistic analysis Data were expressed as mean±SD. The data were analyzed by ANOVA for multiple groups. The significance level was chosen as $P<0.05$.

RESULTS

Vasoconstrictor and vasodilator responses Phenylephrine was added to increase smooth muscle tone in the rat aortic rings. Contraction responses to phenylephrine (1×10^{-6} mol/L) were also significantly increased in the rats treated with LDL. The tension was 1.69±0.11 and 1.22±0.12 g for LDL and control, respectively (n=10-12, $P<0.01$). Pretreatment with LDL (4 mg/kg) for 48 h significantly decreased relaxation responses to acetylcholine. Aspirin (30 or 100 mg/kg) or vitamin E (100 mg/kg) significantly attenuated inhibition of relaxation responses to ACh by LDL (Fig 1).

Concentrations of ADMA Pretreatment with LDL for 48 h significantly increased the serum level of ADMA. Aspirin (30 mg/kg) or vitamin E (100 mg/kg) markedly inhibited the elevated concentration of ADMA by LDL. Aspirin at higher concentrations (100 mg/kg) had no effect on the increased level of ADMA by ox-LDL (Tab 1).

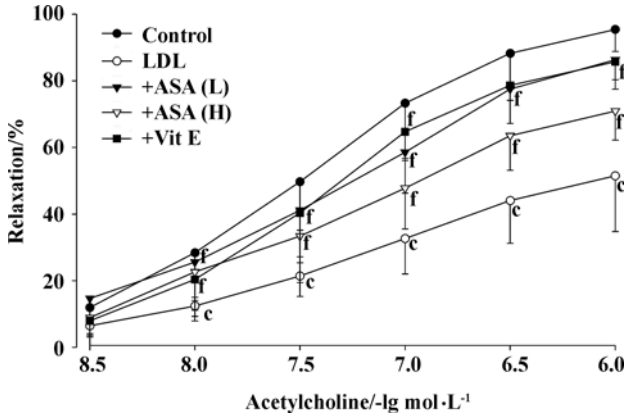

Fig 1. Effect of aspirin on vasodilator responses to acetylcholine. ASA (L): aspirin (30 mg/kg); ASA (H): aspirin (100 mg/kg). *n*=10-12. Mean±SD. $^{c}P<0.01$ *vs* control. $^{f}P< 0.01$ *vs* LDL.

Tab 1. Effect of aspirin on the serum levels of ADMA, MDA, and TNF-α and the activity of DDAH. Aspirin (L): aspirin (30 mg/kg); Aspirin (H): aspirin (100 mg/kg). *n*=10-12. Mean±SD. $^{c}P<0.01$ *vs* control. $^{f}P<0.01$ *vs* LDL.

	ADMA/ μmol·L^{-1}	DDAH/ %	MDA/ nmol·L^{-1}	TNF-α/ μg·L^{-1}
Control	1.07±0.22	100±30	4.0±0.9	2.14±0.27
LDL	1.6±0.4^{c}	48±16^{c}	9±4^{c}	4.3±0.4^{c}
+Aspirin (L)	1.2±0.4^{f}	68±37^{f}	4.6±1.6^{f}	2.5±0.4^{f}
+Aspirin (H)	1.4±0.4	53±42	5.7±1.2^{f}	2.7±0.6^{f}
+Vitamin E	1.2±0.3^{f}	70±23^{f}	5.1±2.0^{f}	2.7±0.6^{f}

Activity of DDAH Pretreatment with LDL for 48 h significantly decreased the activity of DDAH. Aspirin (30 mg/kg) or vitamin E (100 mg/kg) markedly attenuated the inhibition of DDAH activity by LDL in red cells. Aspirin at higher concentrations (100 mg/kg) had no effect on the decreased activity of DDAH by ox-LDL (Tab 1).

Serum concentrations of MDA Pretreatment with LDL caused a significant increase in concentrations of MDA. Aspirin (30 or 100 mg/kg) significantly inhibited the elevated concentration of MDA by LDL. The increased level of MDA by LDL was also inhibited by pretreatment with vitamin E (Tab 1).

Concentrations of TNF-α After pretreatment with LDL for 48 h, serum concentrations of TNF-α were significantly increased. Aspirin (30 or 100 mg/kg) markedly inhibited the elevated concentration of TNF-α by LDL. Vitamin E (100 mg/kg) also markedly inhibited the elevated concentration of TNF-α by LDL (Tab 1).

Expression of ICAM-1 The immunostaining of ICAM-1 was mainly shown in the endothelium. There was a visually perceptible increase in the ICAM-1 content in the LDL-treated rats compared with the control, which was markedly attennated by treatment with aspirin or vitamin E. The reduction expression of ICAM-1 by aspirin at the lower dose was greater compared with the high dose (Fig 2).

Effect of aspirin on DPPH radicals No change in the absorbance of blank solution of DPPH at 517 nm was observed during a period of 5 min. Addition of 3×10^{-5}-1×10^{-2} mol/L aspirin or vitamin E to the DPPH solution caused a dose-dependent inhibition in absorbance at 517 nm. The ED_{50} of aspirin or vitamin E was 100 and 26.9 μmol/L, respectively.

DISCUSSION

Ox-LDL exhibits numerous biological effects, including endothelial dysfunction, activation of endothelial adhesiveness, monocyte differentiation and adhesion, and smooth muscle cell proliferation, suggesting that ox-LDL plays a key role in the development of atherosclerotic lesions[16,17]. The results of the present study confirmed previous observations that a single injection of native LDL caused a significant decrease in endothelium-dependent relaxation to ACh in the aortic ring[7]. In the present study, the effects of LDL and bovine serum albumin on vasodilator responses to ACh were compared. The results revealed that bovine serum albumin did no affect vasorelaxation (data not shown), suggesting that endothelium injury elicited by exogenous LDL is due to its direct toxicity rather than anaphylactic reaction.

NO, an important local regulatory factor in cardiovascular tissues, is synthesized from *L*-arginine by NOS in endothelial cells. NO possesses complex cardiovascular actions such as regulating vascular smooth muscle tone, protecting endothelial cells, and inhibiting vascular smooth muscle cell proliferation[18]. *L*-arginine analogues such as ADMA, which is present in blood of both humans and animals, can inhibit NOS *in vivo* and *in vitro*[19,20]. There is substantial evidence that endothelium dysfunction in the hypercholesterolemic animal and human is associated with elevation of ADMA, and endogenous inhibitors of NOS have been thought as a novel predictor of endothelium dysfunction. It has been

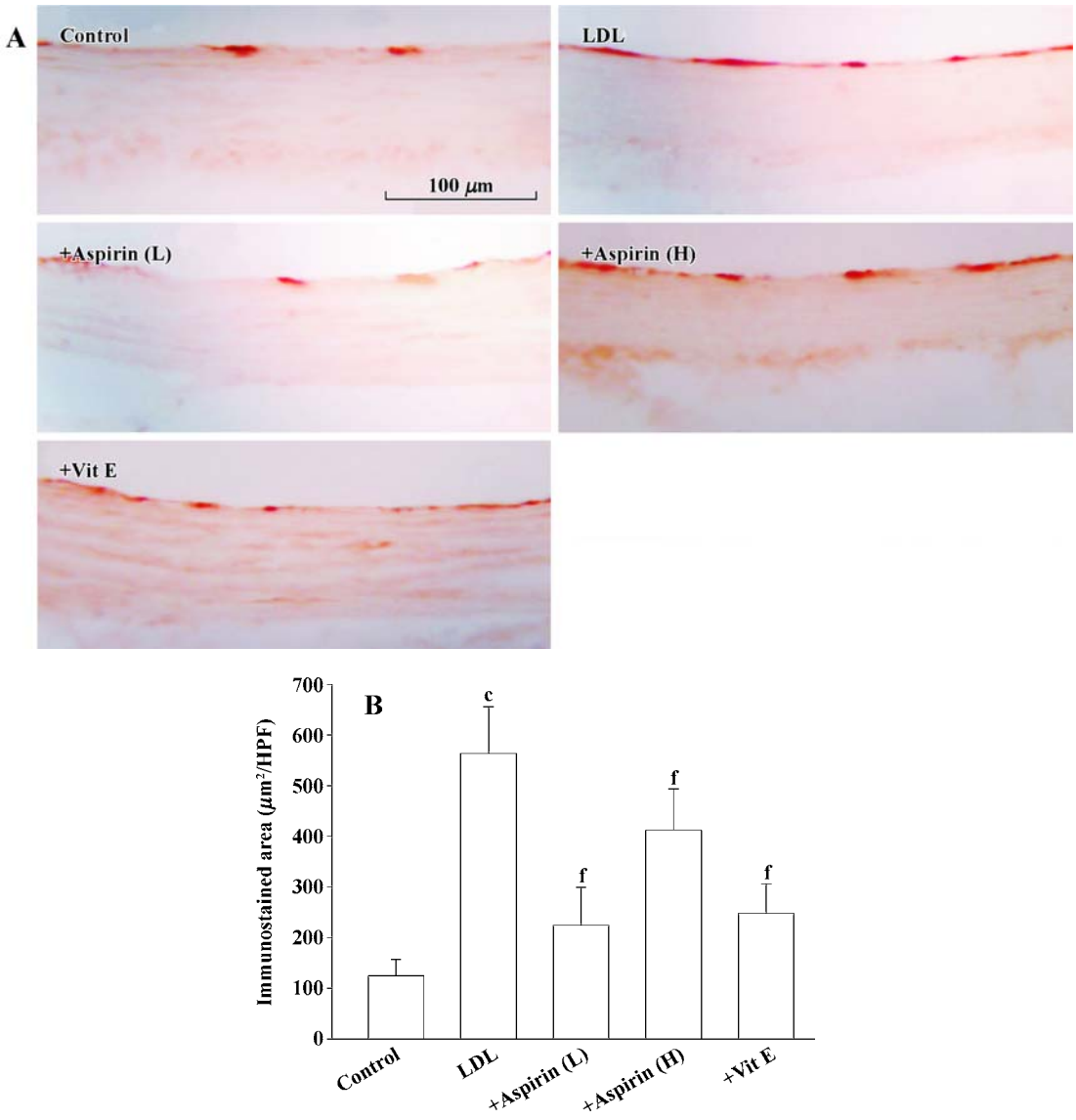

Fig 2. Effect of aspirin on the expression of ICAM-1 in the aorta endothelium. (A) ICAM-1 immunostaining in the aorta ring. The immunoreactive products were mainly shown in the endothelium. The size bar corresponds to 100 μm. (B) Bar graph shows the ICAM-1 immunostained area per high power field (HPF). Aspirin (L): aspirin (30 mg/kg); Aspirin (H): aspirin (100 mg/kg). *n*=10-12. Mean±SD. $^{c}P<0.01$ *vs* control. $^{f}P<0.01$ *vs* LDL.

shown that the increased adhesiveness of monocyte is associated with elevation of serum ADMA level in hypercholesterolemic humans[2,21]. In the present study, pretreatment with LDL significantly increased ICAM-1 content in the endothelium[22]. In cultured endothelial cells, ADMA increased adhesion of monocytes, in further support of the hypothesis that ADMA is a contributor to adhesion of monocytes to endothelial cells[1].

ADMA is synthesized by protein arginine methyltransferases (PRMTs), which utilizes *S*-adenosyl-methionine methyl group donor, and degraded by DDAH, which hydrolyzes ADMA to *L*-citrulline and dimethylamine asymmetric dimethylarginine[23]. ADMA and DDAH are widely distributed in tissues including endothelial cells[24]. There is evidence that lipid-induced dysregulation of DDAH may be an important factor contributing the elevation of ADMA in hypercholesterolemia and hyperhomocysteinemia[25]. Others have been reported that the elevated content of ADMA by LDL or ox-LDL is also related to decrease of DDAH activity in cultured endothelial cells[23].

Inflammatory cytokines such as TNF-α are significantly increased in animals and patients with hypercholesterolaemia[26]. TNF-α is widely synthesized

in various cells including macrophages and endothelial cells. TNF-α has been found to upregulate the expression of ICAM-1 and increase the adhesion of monocyte to endothelium[27]. Recently, it has been reported that TNF-α also elevates the level of ADMA with the decreased activity of DDAH, which hydrolyzes ADMA to *L*-citrulline, in cultured human endothelial cells. The present results revealed that treatment with LDL significantly increased the levels of TNF-α and ADMA in cultured endothelial cells[23]. These results support the conclusion that the increased level of ADMA induced by LDL may be related to reduction of DDAH activity by stimulation of TNF-α production.

Aspirin has extensive pharmacological actions including antioxidation and anti-inflammation. Studies in animal experiments have shown that aspirin has a protective effect on endothelium cells, and the benefitial effect of aspirin on endothelium cells is ascribed to its antioxidant properties, including the inhibition of LDL oxidation and scavenging oxygen free radicals[9-11]. In the present study, aspirin scavenged DPPH radicals *in vitro* and decreased the elevated content of MDA by LDL *in vivo*.

A major finding in the present study was that aspirin attenuated endothelial dysfunction injury concomitantly with a decrease of ADMA level and an increase of DDAH activity in the rat treated with LDL. As mentioned above, ox-LDL and TNF-α can induce ADMA production by reduction of DDAH activity. In the present experiment, aspirin decreased ADMA content and increased DDAH activity, concomitantly with a decrease in the level of MDA and TNF-α. These findings suggest that the productive effect of aspirin on endothelial cells may be related to reduction of ADMA by inhibition of TNF-α production.

In summary, aspirin preserves the vascular endothelium in the rats treated with LDL, and the protective effect is related to reduction of ADMA concentration via increasing DDAH activity.

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