

# DOWNEXPRESSION AND DYSFUNCTION OF ENDOTHELIAL NITRIC OXIDE SYNTHASE ON AORTA BY EXOGENOUS LDL CAN BE TRANSIENTLY RESTORED BY ONE BOUT OF ACUTE EXHAUSTIVE EXERCISE

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Oxidized low-density lipoprotein (oxLDL) formation occurs in the arterial wall and is believed to play a role in atherogenic events. Based on evidence from many cellular and animal studies, endothelial nitric oxide synthase (eNOS) expression is sensitive to oxLDL and exercise with adverse regulatory effect. To study the effect of oxLDL on eNOS protein expression *in vivo*, we followed a well-established animal model in which 24 Wistar rats were treated with intraperitoneal injection of human native LDL (nLDL) or oxLDL (4 mg kg<sup>-1</sup>). At the end of 12 hours of treatment, aortic eNOS protein was significantly decreased by 66% in nLDL-treated rats, and by 82% in oxLDL-treated rats ( $p < 0.01$ ). In striking contrast, one bout of exhaustive treadmill exercise (22.10 ± 1.41 minutes), performed in the 11<sup>th</sup> hour of treatment, restored aortic eNOS protein expression by 86% and 34% in nLDL- and oxLDL-treated rats, respectively ( $p < 0.05$ ). Furthermore, NOS activity and heat shock protein 90 (HSP90) expression correlated with eNOS expression, indicating that HSP90 activated by acute exhaustive exercise may be associated with transient recovery of eNOS and NOS activity. We conclude that downexpression and dysfunction of eNOS caused by exogenous LDL can be transiently restored by one bout of acute exhaustive exercise and may be through association and protection from HSP90.

**Keywords:** endothelial nitric oxide synthase, exhaustive exercise, heat shock protein 90, oxidized low-density lipoprotein

## Introduction

Oxidized low-density lipoprotein (oxLDL) is the most well-studied form of modified LDL and is believed to exert an important role in atherosclerotic lesions (Steinberg et al. 1989). In addition to promoting the formation of

a broad spectrum of biologically active products, such as malondialdehyde, oxysterols, and fragmentation of apolipoprotein B (apoB), oxLDL also impairs vasodilatation of endothelium (Kugiyama et al. 1990), activation of endothelial adhesiveness (Frostegard et al. 1991), and monocyte differentiation (Frostegard et al. 1990), leading to development of early atherosclerotic lesions.

Calara et al. (1998) have established a simple animal model to study the biologic effects of oxLDL accumulated on the arterial wall *in vivo*. As they reported, epitopes specific for human apoB begin to accumulate in the aorta within 2–6 hours after intraperitoneal injection of

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human native LDL (nLDL) ( $4 \text{ mg kg}^{-1}$ ), followed by peak expression of oxLDL at 12 hours. Endothelial activation of transcription factor, nuclear factor- $\kappa$ B, tumor necrosis factor- $\alpha$ , and adhesion molecule-1 is associated with the LDL accumulation event (Niemann-Jonsson et al. 2000; Calara et al. 1998).

In a study of cultured endothelial progenitor cells, oxLDL has been found to exhibit an inhibitory effect on endothelial nitric oxide synthase (eNOS) through dose-dependent decrement of Akt phosphorylation, eNOS protein, and mRNA expression (Ma et al. 2006). Besides, caveolin and eNOS, which forms a caveolin activation complex localized to cholesterol rich cell surface caveolae, translocate to intracellular membrane compartments through a cholesterol depletion mechanism mediated by oxLDL, but not nLDL or high-density lipoprotein (HDL) (Shaul 2003; Everson & Smart 2001; Blair et al. 1999).

Regulation of eNOS activation includes protein-protein interaction with caveolin-1 and -3 (Feron et al. 1996; Garcia-Cardena et al. 1996), the intracellular domain of certain G-protein coupled receptors (Marrero et al. 1999), calmodulin, and heat shock protein 90 (HSP90) (Garcia-Cardena et al. 1998). Caveolin-1 and -3 are reported to show an inhibitory effect in eNOS translocation, whereas calmodulin and HSP90 are stimulatory (Gratton et al. 2000; Rizzo et al. 1998; Ju et al. 1997).

HSP90 has been reported to associate with and thus activate eNOS in a stimulus-dependent manner such as shear stress (Garcia-Cardena et al. 1998), and be uncoupled with eNOS if treated with oxLDL (Stepp et al. 2002).

The primary aim of this study was to test the hypothesis that exogenous nLDL or oxLDL causes downregulation and dysfunction of aortic eNOS protein, and the secondary aim was to examine the possible role of HSP90 in the regulation of eNOS expression.

## Methods

### *Animal protocol*

Twenty-four 8-week-old male Wistar rats were obtained from the Experimental Animal Center of National Taiwan University. All rats were fed with standard chow and water. After familiarizing to treadmill for 3 days, the rats were randomly divided into control (C), nLDL-treated (nL), oxLDL-treated (oL), acute exhaustive exercise (A),

nLDL-treated combined with acute exhaustive exercise (nLA), and oxLDL-treated combined with acute exhaustive exercise (oLA) groups. Each type of LDL treatment was given by intraperitoneal injection  $4 \text{ mg kg}^{-1}$  to rats in conscious state, while vehicle control was 0.9% NaCl. In the nLA and oLA groups, one bout of acute exhaustive exercise was conducted at 11 hours after nLDL and oxLDL treatment, respectively. The acute exhaustive exercise was performed during the active period of rats. The rats ran on a motor-driven treadmill at  $21.7 \text{ m min}^{-1}$  (uphill 27% gradient) for the first 15 minutes. After that, the speed was increased gradually to  $26.7 \text{ m min}^{-1}$  (32% gradient) and kept constant until the rats were exhausted. The loss of the righting reflex when the rats were turned on their backs was the criterion for exhaustion. Rats were sacrificed either 12 hours after nLDL/oxLDL treatment or within 10 minutes after exhaustive exercise. The animal experiments were approved by the Animal Committee of National Dong Hwa University, Taiwan.

### *Preparation and oxidation of LDL*

Blood samples for LDL preparation were provided by the Blood Donation Center, Hualien, Taiwan. Five male volunteers aged 18–40 years were screened in accordance with the criteria of hyperlipidemia. All subjects were non-smokers and free of infectious diseases at the time of sampling. LDL was prepared by sequential ultracentrifugation in a density gradient as described previously with some modifications (Havel et al. 1955). Briefly, LDL was concentrated by pooling LDL prepared from donors, adjusting the density to  $1.075 \text{ g mL}^{-1}$  by adding NaBr containing 0.1% EDTA (pH 7.4) and ultracentrifuging the samples at  $105,000g$  for 20 hours at  $4^\circ\text{C}$ . The top layer was collected and then the density was adjusted to  $1.015 \text{ g mL}^{-1}$  using 0.9% NaCl (pH 7.4). Ultracentrifugation was repeated and the bottom layer was collected, namely nLDL. The protein content was determined as previously described (Lowry et al. 1951). nLDL ( $20 \text{ mg mL}^{-1}$ ) was oxidized by exposure to  $2 \text{ mM CuSO}_4$  for 14 hours at  $37^\circ\text{C}$ . The oxidation state was analyzed by malondialdehyde (MDA) determination (MDA-586™; OXIS Health Products Inc., Portland, OR, USA). To remove salt and  $\text{Cu}^{2+}$ , LDL fraction was dialyzed extensively at  $4^\circ\text{C}$  against 0.9% NaCl (pH 7.4) containing 0.1% EDTA for 3 days or was then frozen in 10% sucrose (v/v) at  $-80^\circ\text{C}$  until use. Within 1 hour before the injection

of nLDL into the animals, sucrose was removed from the LDL by running the samples over a PD-10 column, preequilibrated in 0.9% NaCl (pH 7.4) (Rumsey et al. 1992), and was sterilized by passing it through a 0.45  $\mu\text{m}$  filter and kept on ice until injected.

### **Tissue collection and Western blot**

Rats were anesthetized with urethane (25% solution in normal saline ip). The thoracic aortas were then removed followed by depriving connective tissue and immediately frozen in liquid nitrogen. The samples were kept at  $-80^{\circ}\text{C}$  until assayed. Frozen aorta was immersed in liquid nitrogen, grinded into powder, and homogenized in a lysis buffer (25 mM Tris-HCl, pH 7.4, 1 mM EDTA, pH 8.0, 1% SDS, 1 mM PMSF, 1  $\times$  proteinase inhibitor cocktail). The extracts were clarified by centrifugation at 10,000g for 5 minutes at  $4^{\circ}\text{C}$ . The protein was quantified using detergent compatible protein assay kit (Bio-Rad Laboratories Inc., Hercules, CA, USA). Aliquots of aorta lysate were separated by SDS-PAGE. Proteins in gel were transferred to methanol-pretreated PVDF membranes in semidry transfer system by 15 V for 15 minutes. The membranes were blocked by incubation in Tris-buffered saline (10 mM Tris, pH 7.5 and 100 mM NaCl) containing 0.1% (v/v) Tween 20 and 5% (w/v) non-fat dry milk for 1 hour, followed by an overnight incubation at  $4^{\circ}\text{C}$  with appropriate antibodies such as anti-eNOS antibody (Affinity BioReagents Inc., Golden, CO, USA), anti-HSP90 (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA), and anti-HSP72 (Santa Cruz Biotechnology Inc.) with dilution ratio of 1:1000. The membranes were washed extensively in Tris-buffered saline containing 0.1% (v/v) Tween 20 before incubation for 1 hour with horseradish peroxidase-conjugated secondary antibody (1:2500) (Amersham Pharmacia Biotech Inc., Piscataway, NJ, USA). The membranes were then washed and developed using enhanced chemiluminescence (ECL) procedures (Amersham Pharmacia Biotech Inc.). The bands on membranes were quantified by densitometry (NTH-SCSA ImageTool version 3.0; The University of Texas Health Science Center, San Antonio, TX, USA).

### **Determination of NOS activity**

NOS activity was determined by a commercially available NOS-detection assay kit (Stratagene Inc., La Jolla, CA, USA). Briefly, the aorta was homogenized in five

volumes of Tris-HCl buffer (25 mM, pH 7.4) containing 1 mM EDTA and 1 mM EGTA, and centrifuged at 10,000g at  $4^{\circ}\text{C}$  for 5 minutes. Ten milliliters of supernatant were added to 40  $\mu\text{L}$  of a reaction mixture containing 25  $\mu\text{L}$  of 2  $\times$  reaction buffer (50 mM Tris-HCl, pH 7.4, 6.0  $\mu\text{M}$  tetrahydrobiopterin [ $\text{BH}_4$ ], 2.0  $\mu\text{M}$  flavin adenine dinucleotide [FAD], 2.0  $\mu\text{M}$  flavin adenine mononucleotide [FMN]), 5  $\mu\text{L}$  of 10 mM NADPH (in 10 mM Tris-HCl, pH 7.4), 6.0  $\mu\text{M}$   $\text{CaCl}_2$ , and 1  $\mu\text{L}$  ( $^{14}\text{C}$ )-L-arginine monohydrochloride (50  $\mu\text{Ci mL}^{-1}$ ) (Amersham Pharmacia Biotech Inc.) in a final volume of 50  $\mu\text{L}$ . Following incubation at  $25^{\circ}\text{C}$  for 60 minutes, the reaction was terminated by the addition of 400  $\mu\text{L}$  of 50 mM HEPES (pH 5.5) buffer containing 5 mM EDTA and 100  $\mu\text{L}$  preequilibrated resin (Dowex AG50WX-8; Supelco Inc., Bellefonte, PA, USA). Then the total reaction mixture was applied onto 1.5 mL spin columns, followed by centrifugation at 10,000g for 30 seconds. ( $^{14}\text{C}$ )-L-Citrulline was quantified by scintillation spectroscopy of 10-mL aliquots of the flow-through. Protein concentration was determined using a Protein Assay Solution (Bio-Rad Laboratories Inc.). NOS activity was expressed in terms of  $\text{fmol mg protein}^{-1} \text{ min}^{-1}$ .

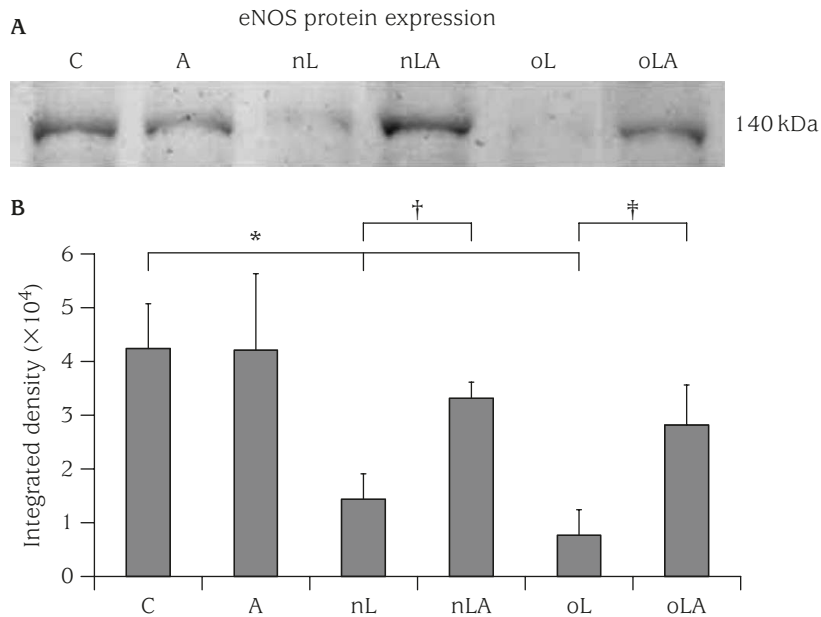
### **Statistical analysis**

Results are expressed as mean  $\pm$  standard deviation. Significant differences between group means were determined by independent one-way analysis of variance followed by Tukey's HSD test. Statistical significance was accepted at  $p < 0.05$ .

## **Results**

### **Regulation of eNOS protein expression by nLDL or oxLDL**

To determine whether exogenous nLDL or oxLDL influences eNOS protein expression, eNOS protein expression was assessed by Western blot. Compared with vehicle control (C group), preinjection with nLDL (nL group) or oxLDL (oL group) 4  $\text{mg kg}^{-1}$  led to significant downexpression of aortic eNOS protein at 12 hours by 66% and 82% respectively (Figure 1). However, one bout of acute exhaustive treadmill exercise at 11 hours partially restored eNOS protein expression by 86% (relative to nL group) and 34% (relative to oL group).



**Fig. 1** Effect of acute exercise on aortic endothelial nitric oxide synthase (eNOS) protein expression in native low-density lipoprotein (nLDL)- or oxidized low-density lipoprotein (oxLDL)-treated rats. (A) Representative Western blot of aortic homogenates in each group, including vehicle control (C), stimulus with acute exercise (A), nLDL (nL) or oxLDL (oL) treatment for 12 hours, stimulus with acute exercise at 11 hours with nLDL (nLA) or oxLDL (oLA) treatment for 12 hours. (B) Densitometric analysis of Western blot. \*Significant difference from C group ( $p < 0.01$ ); †significant difference from nL group ( $p < 0.05$ ); ‡significant difference from oL group ( $p < 0.05$ ).

### Regulation of HSP expression by nLDL or oxLDL

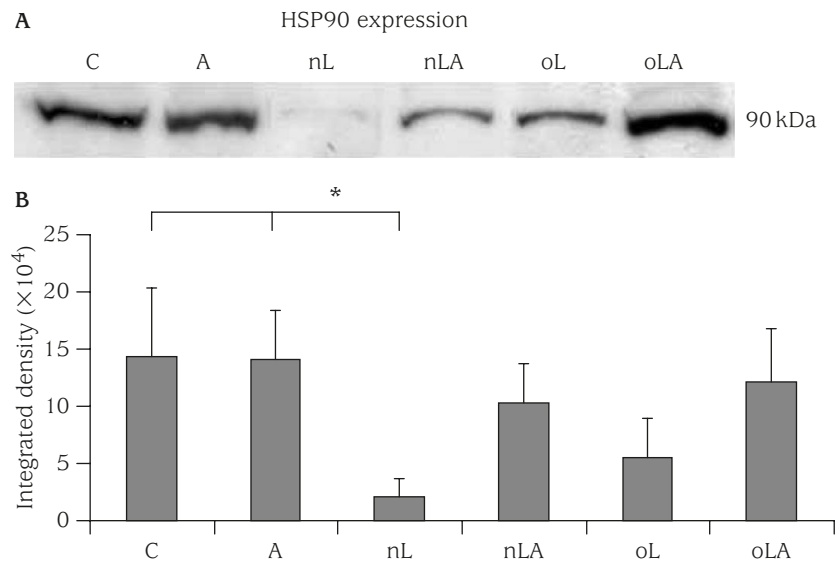
Similar to the eNOS protein expression pattern, HSP90 expression was significantly downregulated by 86% and 62% in the nL group and oL group, respectively, and partially restored by one bout of acute exhaustive treadmill exercise at 11 hours, with an increase of 67% (relative to nL group) and 75% (relative to oL group) (Figure 2). In contrast to changes in HSP90 expression, HSP72 was not affected by injected nLDL or oxLDL (Figure 3).

### Regulation of NOS activity by nLDL or oxLDL

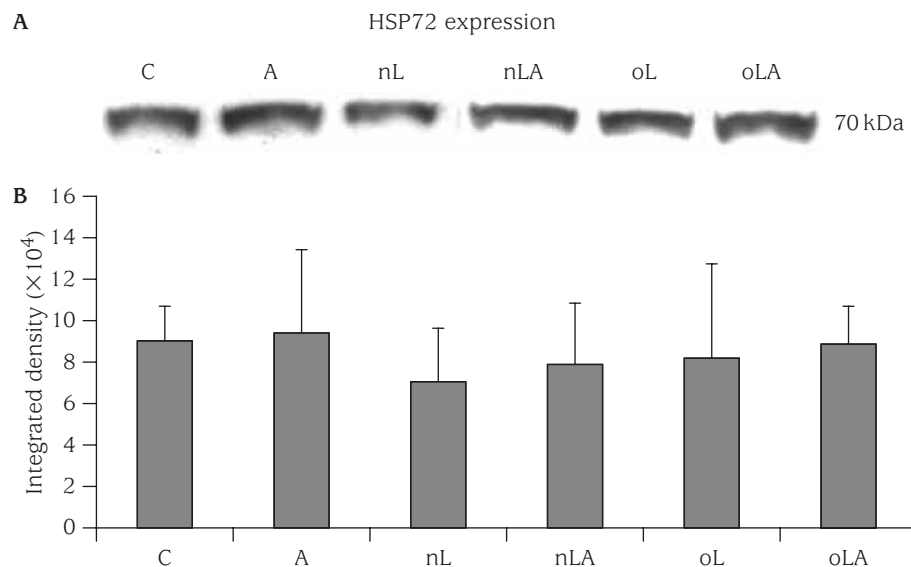
To examine the effect of injected nLDL and oxLDL on endothelial function of the aorta, NOS activity was measured *in vitro*. As shown in Figure 4, NOS activity was significantly reduced by preinjected nLDL and oxLDL by 78% and 68%, respectively. As expected, in the presence of acute exhaustive exercise, NOS activity was significantly restored by 102% (relative to nL group) and 58% (relative to oL group). However, one bout of acute exhaustive exercise did not affect NOS activity.

### Discussion

Evidence from cell culture and animal studies strongly indicates that oxidative modification of LDL plays a significant role in atherogenesis. Limited knowledge of the biologically relevant effect of oxLDL in the arterial wall has been elucidated. A well-established animal model developed by Calara et al. (1998) shows that an accumulation of apoB and epitopes present on oxLDL is detected in the arterial endothelium and media within 6 hours, suggesting potential to impair endothelial function. Other similar studies also demonstrate that LDL particles can be entrapped and aggregated in the subintimal extracellular matrix 2 hours after bolus infusion of heterologous LDL in rabbits, and transendothelial transport of LDL occurs after LDL injection in rats (Lin et al. 1989). Our results support the hypothesis and extend the previous finding. The significant downexpression of eNOS protein of aorta in nLDL or oxLDL-treated rats (Figure 1) ( $p < 0.01$ ) and the fact that caveolin and eNOS translocated from caveolae to an intracellular membrane compartment in oxLDL, but not nLDL (Blair



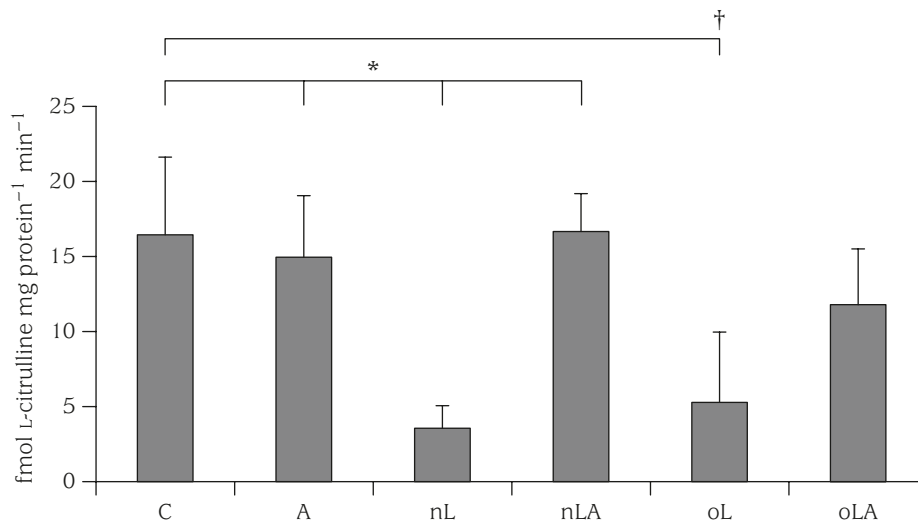
**Fig. 2** Effect of acute exercise on aortic heat shock 90 (HSP90) expression in native low-density lipoprotein (nLDL)- or oxidized low-density lipoprotein (oxLDL)-treated rats. (A) Representative Western blot of aortic homogenates in each group, including vehicle control (C), stimulus with acute exercise (A), nLDL (nL) or oxLDL (oL) treatment for 12 hours, stimulus with acute exercise at 11 hours with nLDL (nLA) or oxLDL (oLA) treatment for 12 hours. (B) Densitometric analysis of Western blot. \*Significant difference from nL group ( $p < 0.01$ ).



**Fig. 3** Effect of acute exercise on aortic heat shock protein 72 (HSP72) expression in native low-density lipoprotein (nLDL)- or oxidized low-density lipoprotein (oxLDL)-treated rats. (A) Representative Western blot of aortic homogenates in each group, including vehicle control (C), stimulus with acute exercise (A), nLDL (nL) or oxLDL (oL) treatment for 12 hours, stimulus with acute exercise at 11 hours with nLDL (nLA) or oxLDL (oLA) treatment for 12 hours. (B) Densitometric analysis of Western blot.

et al. 1999), indicate that downregulation of eNOS protein expression may associate with oxLDL formation and dislocation from cholesterol-rich caveolae. Furthermore, the noxious effect of oxLDL on endothelial progenitor cells includes reduction of eNOS protein, mRNA

expression, and activity in a dose-dependent relationship and may be mediated by upregulation of a lectin-like oxLDL receptor (LOX-1) (Ma et al. 2006). Besides, in agreement with NOS activity being regulated by Akt phosphorylation, which can be reduced by oxLDL, NOS



**Fig. 4** Effect of acute exercise on aortic nitric oxide synthase activity in native low-density lipoprotein (nLDL)- or oxidized low-density lipoprotein (oxLDL)-treated rats: vehicle control (C), stimulus with acute exercise (A), nLDL (nL) or oxLDL (oL) treatment for 12 hours, stimulus with acute exercise at 11 hours with nLDL (nLA) or oxLDL (oLA) treatment for 12 hours. \*Significant difference from nL group ( $p < 0.05$ ); †significant difference from oL group ( $p < 0.05$ ).

activity in nLDL- or oxLDL-treated rats was significantly reduced (Figure 4) ( $p < 0.05$ ). Previous studies have shown that depletion of membrane cholesterol impairs caveolae structure, induces eNOS translocation from caveolae to intracellular locations, and thus inhibits eNOS activity (Zhang et al. 2006).

HSP90 has been demonstrated to associate with eNOS and is rapidly recruited to the eNOS complex by agonists that stimulate production of nitric oxide such as vascular endothelial growth factor, histamine, and shear stress (Garcia-Cardena et al. 1998). Exposure of endothelial cells to fluid shear stress ( $15 \text{ dynes cm}^{-2}$ ) stimulates the association of eNOS and HSP90, with a constant total amount of cellular HSP90, suggesting that HSP90 exerts a role on eNOS activation to nitric oxide production (Garcia-Cardena et al. 1998). Both nLDL and minimally oxidized LDL (mmLDL) uncouple eNOS activity by disrupting HSP90 and eNOS interaction with increased eNOS-dependent superoxide anion production (Stepp et al. 2002). Considering this evidence and our results, the reduction in eNOS protein expression was accompanied by decreased HSP90 but not HSP72 expression (Figures 1–3), indicating the putative protective role of HSP90 was required for full eNOS protein expression and activity. In contrast to a damaging effect from oxLDL, HDL has been shown to play a role of antagonist for oxLDL. HDL prevented the depletion of caveolae

cholesterol by oxLDL and also inhibited the translocation of eNOS and caveolin from the cell surface. Several receptors that mediate lipoprotein binding has been shown to be located on caveolae, indicating that they may be involved in the efflux mechanism of caveolar cholesterol (Uittenbogaard et al. 2000, 1998; Tall 1990).

As mentioned earlier, the eNOS activation mechanism involves association with calmodulin or HSP90. While activated by ligand or mechanotransduction pathways, calmodulin associates with eNOS to dissociate from caveolin, which is facilitated in the presence of HSP90 (Gratton et al. 2000). The association between HSP90 and eNOS can be initiated only within 15–30 minutes under fluid shear stress ( $15 \text{ dynes cm}^{-2}$ ). This rapid stimulus-dependent formation of the eNOS–HSP90 heterocomplex indicates that the interaction proceeds or occurs simultaneously with other signaling events, such as calcium activation of other downstream effectors required nitric oxide release (Garcia-Cardena et al. 1998). In our model, the stimulus of one bout of acute exhaustive treadmill exercise mimicked that of strong shear stress and significantly restored the eNOS protein expression and activity of aorta in nLDL- and oxLDL-treated rats (Figures 1 & 4). Similar to the effect on eNOS protein expression, but not statistically significant, HSP90 expression was restored partially in nLDL- and oxLDL-treated rats (Figure 2). In contrast to the

effect of acute exercise on HSP90, HSP72 protein expression appears not be affected (Figure 3). HSP72 expression has been known to be induced in different tissues, including skeletal muscle, heart, liver and brain (Fehrenbach & Niess 1999). Though 1 hour of acute treadmill exercise significantly activated the synthesis and accumulation of hepatic HSP72 (Gonzalez & Manso 2004), in this study, aortic HSP72 expression was not affected by acute exercise, neither HSP90 nor eNOS protein expression. Tissue difference and/or exercise duration may be the possible causes. More detailed analysis is necessary to elucidate the precise mechanism.

In summary, we applied an animal model to test the hypothesis that downexpression and dysfunction of eNOS protein resulting from injected nLDL or oxLDL can be significantly restored by exercise. It is unlikely the results can be explained by translational or transcriptional modification, considering the short time span. Indirect results indicate that HSP90 may play a protective role to activate eNOS. The present study shows that vascular health can be transiently improved by acute exercise. Further studies may extend the animal model to explore the molecular mechanism about oxLDL formation *in vivo* and if/how chronic exercise training could attenuate the cytotoxicity of oxLDL.

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