Chronic Treatment with α-Lipoic Acid Improves Endothelium-Dependent Vasorelaxation of Aortas in High-Fat Diet-Fed Mice

Yasuhiro Takenouchi,*a,b Kazuhiro Tsuboi,a Kenji Ohsuka,b Koji Nobe,c Kazuo Ohtake,b Yasuo Okamoto,a and Keizo Kasono*b

*a Department of Pharmacology, Kawasaki Medical School; 577 Matsushima, Kurashiki, Okayama 701–0192, Japan:
b Laboratory of Physiology, Faculty of Pharmaceutical Sciences, Josai University; 1–1 Keyakidai, Sakado, Saitama 350–0295, Japan; and *Division of Pharmacology, Department of Pharmacology, Toxicology and Therapeutics, School of Pharmacy, Showa University; 1–5–8 Hatanodai, Shinagawa-ku, Tokyo 142–8555, Japan.

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α-Lipoic acid (ALA) is used as a dietary supplement and known as an anti-oxidant. The present study aimed to examine whether ALA improves endothelial dysfunction in high-fat diet-fed obese mice. After feeding a high-fat diet to Institute of Cancer Research (ICR) mice for 4 weeks, the mice were maintained with a high-fat diet (group HF) or a high-fat diet containing ALA (25 mg/d, group HF + ALA) for an additional 20 weeks. Age-matched normal diet-fed mice were also used (group Normal). Chronic oral treatment with ALA did not affect various plasma parameters or body weights. As compared with the aortas of Normal mice, those from HF mice showed impaired endothelium-dependent relaxation in response to clonidine. However, such an impairment was not observed in the aortas from HF + ALA mice. The plasma levels of thiobarbituric acid-reactive substances, an indicator of oxidative stress, were significantly decreased in HF + ALA mice compared with HF mice, confirming the anti-oxidative effects of ALA. In addition, when the impaired clonidine-induced vasorelaxation of aortas from normal mice under high glucose conditions was used as a model of acute oxidative stress, the vasorelaxation responses were improved in the presence of ALA at 100 µM. Our results suggested that the chronic oral administration of ALA improves endothelial dysfunction in high-fat diet-fed obese mice possibly through the reduction in oxidative stress in vivo.

Key words α-lipoic acid; obesity; endothelial dysfunction; thoracic aorta; oxidative stress; vasorelaxation

INTRODUCTION

Obesity is a dominant risk factor for the development and progression of diabetes, hypertension, dyslipidemia, atherosclerosis, and other life-threatening cardiovascular diseases. Both animal and clinical studies have indicated that obesity is closely associated with vascular dysfunction followed by prominent atherosclerosis. The recent adoption of high-fat diets has contributed to the increased incidence of cardiovascular diseases such as hypercholesterolemia and hyperglycemia. Obesity causes increases in the blood levels of glucose, inflammatory cytokines, and reactive oxygen species as well as a decrease in nitric oxide (NO) levels, all of which have been reported to lead cardiovascular diseases. However, the mechanisms underlying obesity-induced cardiovascular diseases are not fully clarified. Thus, its better understanding is imperative for the establishment of the future therapies to reduce morbidity and mortality of cardiovascular events.

Endothelial dysfunction is considered to occur at an early step in the development of cardiovascular diseases such as atherosclerosis. Since endothelium-derived NO plays a significant role in the vasodilation, endothelial dysfunction is regarded as a cardiovascular risk factor, especially in high-risk subjects. Furthermore, the improvement of the endothelial dysfunction could reduce the cardiovascular risk. Although there have been many studies concerning the cause of endothelial dysfunction, oxidative stress is strongly suggested to be the most important factor as a direct inducer of endothelial dysfunction.

α-Lipoic acid (ALA) is used as a dietary supplement to support anti-aging, weight reduction, exercise performance, and recovery from exhaustion. ALA is a necessary cofactor for the metabolisms of carbohydrates, proteins, and fats, and also for the conversion of energy to ATP. Most importantly, ALA has been shown to have an anti-oxidant effect both in vitro and in animal experiments, and this effect may contribute to the improvement of endothelial dysfunction. ALA was also reported to have defensive effects on several stress related with endothelial dysfunction in type 2 diabetes, hyperruricemia, cerebral ischemia and H2O2-exposed conditions. However, the effects of prolonged treatment with ALA on vascular function in obese mice have not been studied. While hypocholesterolemic drugs such as statins are known to protect against cardiovascular diseases, the consumption of functional foods or dietary supplements to reduce cardiovascular disease risk is widely accepted by the general public, justifying the use of ALA rather than medicines. In the present study, we examined the effects of long-term oral supplementation of ALA on endothelial function in thoracic aortas in high-fat diet-induced obese mice.

MATERIALS AND METHODS

Reagents Prostaglandin F2α (PGF2α) was purchased from Ono Pharmaceuticals (Osaka, Japan). Sodium nitroprusside (SNP) and LY294002 were from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Clonidine hydrochloride, phenylephrine hydrochloride (Phe), yohimbine, and Nnitro-L-
arginine methyl ester (l-NAME) were from Sigma-Aldrich (St. Louis, MO, U.S.A.). The chloride salt of acetylcholine (ACh) was from Daiichi Pharmaceuticals (Tokyo, Japan). ALA was from Tokyo Chemistry Industry (Tokyo, Japan). For the measurements of vascular reactivity, ALA and LY294002 were dissolved in methanol, respectively, and the methanol solutions were diluted with an aqueous buffer to make desired concentrations. The final concentration of methanol was adjusted to 0.1%. All the other reagents were dissolved in saline.

Preparation of Animals Institute of Cancer Research (ICR) male mice (Tokyo Animal Laboratories, Tokyo, Japan) at 4 weeks of age were allowed to acclimate for 1 week with a standard diet (CE2; CLEA, Tokyo, Japan). Mice were housed on a 12-h dark/light cycle at 22 ± 2°C and 55 ± 10% humidity. In order to induce obesity, the mice were fed a high-fat diet containing lard oil (38.9% energy as fat) for 4 weeks. The other group was fed with the same high-fat diet containing 15.1% energy as fat. For further 20 weeks, one group was continued to maintain the high-fat diet (HF diet) and the other group was fed with the same high-fat diet containing ALA (25 mg/d, group HF + ALA). Age-matched ICR mice were used as normal mice (group Normal). Mice were given 5 g/d/mouse of a standard diet, HF diet and HF diet containing ALA. ALA was powdered and mixed into HF diet at 0.5% (w/w). For Figs. 2 and 5, ICR male mice were fed a CE2 diet and used at 6 weeks old. All animal experiments were performed according to the Guide for the Care and Use of Laboratory Animals published by The National Academies Press and approved by the Institutional Animal Care and Use Committee of Josai University and the Animal Research Committee of Kawasaki Medical School, which conform to US National Institutes of Health guidelines.

Preparation of Arterial Rings Thoracic aortas were extracted from animals immediately after anesthetization, placed in an ice-cold Krebs–Henseleit solution (KHS; 118 mM NaCl, 4.7 mM KCl, 25 mM NaHCO3, 1.8 mM CaCl2, 1.2 mM NaH2PO4, 1.2 mM MgSO4, and 11.1 mM glucose), and excised to make rings of 3 mm in length. The rings were then fixed with stainless fine needles at both ends and trimmed free of adherent fat and connective tissue. For some experiments, the endothelium was mechanically removed from the rings by gently rolling the lumen of the vessel on a thin wire.

Measurement of Plasma Levels of Glucose, Total Cholesterol, Triglyceride, Insulin, and Thiobarbituric Acid Reactive Substances (TBARS) Plasma parameters were measured as described previously. Briefly, blood samples were centrifuged (900 × g for 20 min at 4°C), and heparin plasma was stored at −20°C until assay. Plasma levels of glucose, total cholesterol, and triglyceride were measured by using commercially available enzymatic kits (Wako Pure Chemical Industries, Ltd.). Plasma insulin levels were determined by an enzyme-linked immunosorbent assay (Shibayagi, Gunma, Japan). Lipid peroxidation in plasma was evaluated by a TBARS assay, a commonly used method (Cayman TBARS Assay Kit, Cayman Chemical, Ann Arbor, MI, U.S.A.).

Measurement of Vascular Reactivity Each aorta ring was placed in a bath containing 10 mL of KHS (containing 11.1 mM glucose, normal glucose) or modified KHS containing 22.2 mM glucose (high glucose) bubbled with 95% O2 plus 5% CO2 at 37°C. As described previously, one end of each ring was connected to a tissue holder and the other to a force-displacement transducer (Nihon Kohden, Tokyo, Japan). In order to evaluate the vascular relaxation, aortic rings were precontracted with PGF2α (10−5, 3 × 10−5 M) or Phe (10−5 M). After the precontraction had reached a plateau, the relaxant agent, ACh (10−9–10−5 M), clonidine (10−7–10−2 M), or SNP (10−10–10−5 M), was added in a cumulative manner. For some experiments, LY294002, yohimbine, and l-NAME were pre-treated 30 min before the precontraction by PGF2α. To examine the effects of ALA (10, 100 μM, and 1 mM) on the responses to the relaxant agents, ALA was directly added to the bath 30 min before the precontraction by Phe.

Western Blotting The extracted aortic tissues were homogenized, and total proteins were isolated using the T-PER Tissue Protein Extraction Reagent (Thermo Fisher Scientific, Waltham, MA, U.S.A.). The proteins were then solubilized with Lane Marker Reducing Sample Buffer (Thermo Fisher Scientific). The protein concentrations were estimated by bicinchoninic acid (BCA) method (BCA protein Assay, Thermo Scientific). The proteins were then solubilized with Lane Marker Reducing Sample Buffer (Thermo Fisher Scientific). The protein concentrations were estimated by bicinchoninic acid (BCA) method (BCA protein Assay, Thermo Fisher Scientific). Proteins (20 μg/lane) were electrophoresed through 12.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels and electrotransferred to polyvinylidene difluoride membranes (Bio-Rad, Hercules, CA, U.S.A.). The detections by antibodies were then performed according to standard protocols. Briefly, after the blocking of the residual protein sites on the membrane, the membrane was incubated with anti-Akt antibody and anti-Ser473-phosphorylated Akt antibody (1:1000; Cell Signaling Technology, Beverly, MA, U.S.A.), or anti-β-actin antibody (1:5000; Sigma-Aldrich) in the blocking solution, followed by the incubation with secondary antibodies. β-Actin was used as a housekeeping control protein for normalization.

Statistical Analysis All values are expressed as the mean ± standard error (S.E.). Statistical differences in were evaluated using one-way of variance ANOVA followed by Tukey–Kramer’s post hoc test. Statistical comparisons of concentration–response curves were performed using two-way repeated measures ANOVA with Tukey–Kramer’s post hoc test. These analyses were conducted using the StatView program (SAS Institute, Cary, NC, U.S.A.). p values less than 0.05 were considered statistically significant.

Table 1. Compositions of Experimental Diets

| Ingredients                  | Normal | HF   | HF + ALA |
|------------------------------|--------|------|----------|
| Corn starch (g)              | 479.5  | 339.5| 334.5    |
| Sucrose (g)                  | 150.0  | 150.0| 150.0    |
| Casein (g)                   | 200.0  | 200.0| 200.0    |
| l-Cysteine (g)               | 3.0    | 3.0  | 3.0      |
| Cellulose (g)                | 50.0   | 50.0 | 50.0     |
| Soybean oil (g)              | 40.0   | 40.0 | 40.0     |
| Lard (g)                     | 30.0   | 160.0| 160.0    |
| Cholesterol (g)              | —      | 10.0 | 10.0     |
| Mineral mix (g)              | 35.0   | 35.0 | 35.0     |
| Vitamin mix (g)              | 10.0   | 10.0 | 10.0     |
| Choline bitartrate (g)       | 2.5    | 2.5  | 2.5      |
| ter-butylhydroquinone (g)    | 0.01   | 0.01 | 0.01     |
| α-Lipoic acid (g)            | —      | —    | 5.0      |
| Total (g)                    | 1000   | 1000 | 1000     |
| kcal                        | 4159.9 | 4859.9| 4859.9  |
| Fat % (Calories)             | 15.1   | 38.9 | 38.9     |
RESULTS

Plasma Levels of Glucose, Insulin, Cholesterol, and Triglyceride In order to examine the effects of chronic oral administration of ALA on obese mice, ICR mice at 5 weeks of age were fed a high-fat diet for 24 weeks to induce obesity. As shown in Table 2, the nonfasting plasma levels of glucose, total cholesterol, and high density lipoprotein (HDL)-cholesterol as well as body weights were significantly elevated in high-fat diet-fed mice (group HF) compared with age-matched normal mice (group Normal). The plasma insulin or triglyceride level was not significantly different between Normal and HF groups. We then examined the effects of chronic ALA treatment by feeding ALA-containing high-fat diet for 20 weeks (group HF + ALA) at 4 weeks after starting the high-fat diet-feeding. The results showed no significant difference in the plasma parameters or body weights between HF and HF + ALA mice. Thus, ALA did not have apparent effects on obesity itself at the dose used in the present study.

Vascular Reactivity in Aortas We next evaluated the possible effects of the chronic ALA administration on the vascular reactivity of aorta. The aortas were isolated from Normal, HF, and HF + ALA mice, and the relaxation activities of ACh, SNP, and clonidine were examined. After the precontraction by PGF$_{2\alpha}$ ($10^{-6}$–$3 \times 10^{-6}$ M) had reached a plateau, ACh ($10^{-9}$–$10^{-5}$ M), SNP ($10^{-10}$–$10^{-5}$ M), or clonidine ($10^{-8}$–$10^{-6}$ M) was cumulatively added. The results showed that the clonidine-induced relaxations of aortic rings from HF mice were significantly weaker than those from Normal mice (Fig. 1C). This impaired relaxation was significantly improved by the chronic ALA administration (HF + ALA mice). In contrast, the relaxation responses induced by ACh or SNP did not differ significantly among the three groups (Figs. 1A, B).

While the vasodilation by SNP is endothelium-independent, that by ACh is principally dependent on NO released from the endothelium. Since only clonidine-induced vasorelaxation was impaired by feeding a high-fat diet and improved by the ALA treatment, we next compared the

Table 2. Various Parameter Values in Normal, HF and HF + ALA Mice

| Parameters         | Normal       | HF           | HF + ALA     |
|--------------------|--------------|--------------|--------------|
| Body weight (g)    | 33.4 ± 2.4   | 60.0 ± 1.8***| 52.6 ± 4.2***NS|
| Glucose (mg/dL)    | 120.0 ± 11.2 | 172.9 ± 23.4**| 224.4 ± 11.5***NS|
| Cholesterol (mg/dL)| 79.7 ± 7.1   | 205.7 ± 12.7***| 170.0 ± 11.9***NS|
| HDL-cholesterol (mg/dL)| 60.4 ± 10.6   | 147.6 ± 12.8**| 107.4 ± 12.8e NS|
| Triglyceride (mg/dL)| 60.5 ± 13.2 | 60.5 ± 9.3   | 78.2 ± 11.8  |
| Insulin (ng/mL)    | 1.8 ± 0.9    | 1.9 ± 0.6    | 2.6 ± 0.7    |

Values are means ± S.E. *p < 0.05, **p < 0.01, ***p < 0.001, vs. Normal mice. NS p/uni2267 0.05 vs. HF mice.

Fig. 1. Concentration-response Curves for ACh- (A), SNP- (B), and Clonidine- (C) Induced Vasorelaxation of Isolated Aortic Rings from Normal, HF, and HF + ALA Mice

Each data point represents the mean ± S.E. of 6 or 7 mice. ***p < 0.001.
endothelium-dependent vasorelaxation pathways initiated by ACh and clonidine. For this purpose we used aortic rings from normal diet-fed mice and analyzed the effects of various inhibitors. As shown in Fig. 2A, the relaxation induced by both ACh and clonidine almost completely disappeared in the presence of L-NAME (an NO synthase inhibitor, 100 µM) and in endothelium-denuded aortic rings, which is consistent with the relaxant mechanism dependent on endothelium-derived NO.

We then investigated the involvements of phosphatidylinositol 3-kinase (PI3K)/Akt/endothelial NO synthase (eNOS) pathway and α₂-adrenergic receptor. The results showed that the vascular relaxation induced by clonidine was significantly attenuated by LY294002 (a PI3K inhibitor, 10 µM) and almost abolished by yohimbine (an α₂-adrenergic receptor antagonist, 1 µM). However, these two inhibitors did not significantly affect the vascular relaxation induced by ACh (Fig. 2B).
Akt Activation in Aortas  In the vascular reactivity analyses of normal mice, clonidine-induced relaxation was attenuated when the PI3K/Akt/eNOS pathway was inhibited. Since clonidine-induced relaxation was impaired in HF mice and recovered in HF + ALA mice, we next examined phosphorylation of Akt in aortas from Normal, HF, and HF + ALA mice. There was no difference in the level of phosphorylation of Akt among Normal, HF, and HF + ALA mice (Fig. 3).

Oxidative Stress in Plasma  Since oxidative stress is closely associated with obesity,3) we next focused on the antioxidant activity of ALA. TBARS assay was used to evaluate the amount of peroxidized lipid, such as malondialdehyde.29) As expected, the concentrations of plasma TBARS in HF mice tended to be elevated compared to Normal mice (Fig. 4). Chronic ALA treatment in HF mice significantly (*p<0.05) reduced plasma concentrations of TBARS (HF + ALA mice), confirming the antioxidant activity of ALA in vivo.

Effects of ALA on Vascular Reactivity of Aortas under High Glucose Condition  High glucose conditions are reported to cause endothelial dysfunction via oxidative stress.20,21) In order to examine whether the anti-oxidant activity of ALA is beneficial in high glucose conditions, aortas were isolated from normal diet-fed mice and subjected to the relaxation experiments. ALA was directly added to the organ bath 30 min before the precontraction by Phe. As expected, the clonidine-induced relaxation observed under a normal glucose condition (11.1 mM) was attenuated under a high glucose condition (22.2 mM) (Fig. 5A). The pretreatment with a low concentration (10 μM) of ALA had essentially no effects on the impaired relaxation responses, which were recovered when higher concentrations (100 μM and 1 mM) of ALA were used (Fig. 5A). This improvement of clonidine-induced vasorelaxation by 100 μM ALA was significantly attenuated by pretreatment with LY294002 (Fig. 5C). The ACh-induced relaxations were not significantly affected in the aorta under the high glucose condition or by its pretreatment with 10 μM ALA (Fig. 5B). ALA had no vasodilation responses at both 100 μM and 1 mM, shown as a force tracing line (Fig. 5D).

DISCUSSION  This study demonstrated that high-fat diet-induced obesity impaired endothelium-dependent vasorelaxation of mouse aortas by clonidine and that chronic oral ALA treatment improved the vasorelaxation. The beneficial effect of ALA was suggested to be due to the direct action to aortas and the non-direct action including the reduction in oxidative stress in vivo. We also found that chronic ALA administration did not affect various plasma parameters or body weight.

When ALA was administered for 20 weeks to high-fat diet-fed mice, there were no significant effects on plasma levels of glucose, total cholesterol, triglyceride, or insulin. Thus, the beneficial effects of ALA were considered to be unrelated to the amelioration of hyperglycemia or hypercholesterolemia.

The present study compared the effects of vasodilators on aortas from high-fat diet-induced obese mice. To discriminate between the endothelium-dependent and -independent relaxations, we used three vasodilators, ACh, clonidine, and SNP (Fig. 1). SNP is an endothelium-independent vasodilator capable of providing a source of NO, whereas the vasorelaxation by clonidine and ACh is endothelium-dependent.22,23) As shown in Fig. 1, the sensitivity of the aortic smooth muscle cells to SNP was not affected in HF mice, while clonidine-induced vasorelaxation, but not ACh-induced one, was impaired in HF mice. Thus, high-fat diet-induced obesity was considered to impair the endothelial signaling pathway for vasorelaxation caused by clonidine, but not ACh. In endothelial cells, the main pathway stimulated by agonists such as ACh and bradykinin involves Ca²⁺/calmodulin-dependent eNOS activation.28) In contrast, the eNOS activation by shear stress,25) insulin,26) and estrogen27) is independent of a rise in intracellular Ca²⁺, and PI3K/Akt pathway has been reported to be involved in such a calcium-independent eNOS activation.28) We also reported that an Akt inhibitor had no significant effect on either ACh-induced vasorelaxation or NOx/cyclic GMP production in normal mouse aortas.29) In contrast, Akt inhibitors completely abolished the clonidine- and insulin-induced vasorelaxation responses as well as NOx/cyclic GMP production.29) Furthermore, α2-adrenergic receptor expressed in endothelial cells of aorta was reported to be related to vascular relaxation, although this receptor is also present in vascular smooth muscle cells and mediates vasoconstriction.30) In the present study, we showed that the endothelium-dependent relaxation by clonidine occurred via α2-adrenergic receptor/PI3K/Akt/eNOS pathway (Fig. 2). The results are consistent with the above-mentioned mechanisms of clonidine-induced vasorelaxation.

The most important finding obtained in the present study was that chronic ALA treatment recovered clonidine-induced vascular relaxation which was impaired in high-fat diet-fed mice. This beneficial effect of ALA treatment was considered to be possibly due to the improvement of the endothelial function. Since it has been reported that ALA has various effects through activation of the PI3K/Akt pathway,31,32) we were interested to examine whether the improvement was due to the repairment of the PI3K/Akt/eNOS pathway. As shown in Fig. 3, Akt phosphorylation did not differ among Normal, HF and HF + ALA. Although the PI3K/Akt pathway activation is thought to be involved in the protective effect of ALA against endothelial dysfunction in vivo, it might be difficult to detect the significant change in the phosphorylation of Akt in isolated aortas from mice, even if the PI3K/Akt pathway in endothelial cells was activated or inhibited. Furthermore,
it has also been previously reported that the products generated by PI3K activation, such as phosphatidylinositol 3,4,5-trisphosphate, bind to various target proteins other than Akt.\textsuperscript{33,34} Further investigation including the possibility of the PI3K-dependent, and Akt-independent, vasoprotective effects induced by ALA is necessary.
It is well documented that oxidative stress induces endothelial dysfunction in diabetes, hypertension, and obesity. Thus, we next investigated the antioxidative effects of ALA as the underlying mechanism to improve endothelial dysfunction. First, we examined the plasma TBARS concentrations (Fig. 4). The measurement of TBARS is well-established as a method for screening and monitoring lipid peroxidation. Lipid peroxidation is known to cause cellular injury and can be used as an indicator of oxidative stress in cells and tissues.

In the present study, the plasma levels of TBARS in HF + ALA mice decreased in comparison with those in the HF group. This result indicated that ALA actually worked as an antioxidant in our current experiment system and suggested that ALA could improve endothelial function through its anti-oxidative effects. The high glucose condition is known to cause endothelial dysfunction via both acute and chronic oxidative stress, which is associated with impaired endothelium-dependent relaxation.

In the present study, we confirmed that the endothelium-dependent relaxation of aortas by clonidine was impaired in the buffer containing a high concentration of glucose as a model of acute oxidative stress (Fig. 5). The high glucose-induced impairment in clonidine-induced vasodilation was reversed in the presence of ALA at 100 µM and 1 mM, but not at 10 µM (Fig. 5). In the pharmacokinetics studies, it was reported that the maximum plasma concentrations in mice after the oral administration of ALA at 50 and 100 mg/kg were 7.6 ± 1.4 µg/mL (around 36 µM) and 30.9 ± 1.4 µg/mL (around 150 µM), respectively, and that the half-life was around 27 min after the oral administration at 20 mg/kg in rats.

Although the actual plasma concentration of ALA in the present study using mice constantly ingesting ALA with the diet was not measured, our results suggested that the mechanisms for the beneficial effects of chronic ALA administration on the clonidine-induced vasodilation could involve the action to aortas. Pretreatment of LY294002 inhibited ALA-enhanced clonidine-induced vasorelaxation under high glucose condition (Fig. 5C), and ALA itself did not have the ability of vasorelaxation as shown in Fig. 5D. Several studies have revealed that exposure of cells to high glucose apparently increased the generation of oxidative stress and impaired the PI3K/Akt/eNOS pathway, and besides, this impairment was improved by ALA and other antioxidants. In Fig. 5C, the aortic vasorelaxation induced by clonidine (at concentrations of 3 × 10⁻¹⁷ and 10⁻⁶ M) was partially resistant to LY294002. This LY294002-insensitive vasorelaxation might be mediated through Ca²⁺/calmodulin/eNOS pathway because it is reported that clonidine and other α₂-adrenergic receptor ligands activate this pathway. These results suggest that PI3K/Akt pathway is more sensitive to oxidative stress than Ca²⁺/calmodulin-dependent pathway, and ALA may rescue impaired PI3K/Akt pathway by the suppression of oxidative stress.

In conclusion, we showed that the impairment of endothelium-dependent relaxation by clonidine in aortas from high-fat diet-induced obese mice was prevented by chronic treatment of the mice with an antioxidant ALA. Although further experiments would be required to examine detailed mechanisms of the useful action of ALA, the present study suggested that chronic ALA treatment may be useful for the reduction of cardiovascular events in the obese patients.

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Conflict of Interest The authors declare no conflict of interest.

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