Objective—Lipid accumulation in vascular endothelial cells may play an important role in the pathogenesis of atherosclerosis in obese subjects. We showed previously that α-lipoic acid (ALA) activates AMP-activated protein kinase (AMPK) and reduces lipid accumulation in skeletal muscle of obese rats. Here, we investigated whether ALA improves endothelial dysfunction in obese rats by activating AMPK in endothelial cells.

Methods and Results—Endothelium-dependent vascular relaxation was impaired, and the number of apoptotic endothelial cells was higher in the aorta of obese rats compared with control rats. In addition, triglyceride and lipid peroxide levels were higher, and NO synthesis was lower. Administration of ALA improved all of these abnormalities. AMPK activity was lower in aortic endothelium of obese rats, and ALA normalized it. Incubation of human aortic endothelial cells with ALA activated AMPK and protected cells from linoleic acid–induced apoptosis. Dominant-negative AMPK inhibited the antiapoptotic effects of ALA.

Conclusions—Reduced AMPK activation may play an important role in the genesis of endothelial dysfunction in obese rats. ALA improves vascular dysfunction by normalizing lipid metabolism and activating AMPK in endothelial cells.

Key Words: α-lipoic acid ■ endothelium ■ AMPK ■ oxidative stress ■ vascular dysfunction

Central obesity is associated with increased cardiovascular morbidity and mortality.1 Subjects with central obesity show increased lipid accumulation in nonadipose tissues such as muscle, liver, and pancreatic islets.2,3 It has been proposed that increased lipid accumulation in vascular tissue and the consequent increase in oxidative stress may be a missing link between obesity and atherosclerosis.4

α-Lipoic acid (ALA), a naturally occurring short chain fatty acid containing sulfhydryl groups, has potent antioxidant capacities. ALA is an essential cofactor for mitochondrial respiratory enzymes and improves mitochondrial function.5,6 We showed in rodents recently that chronic ALA treatment significantly reduced body weight gain primarily by decreasing food intake, and that this effect was mediated by the effect of ALA to decrease AMPK-activated protein kinase (AMPK) activity in the hypothalamus.7 AMPK is a major regulator of cellular energy metabolism.8,9 When activated, AMPK increases glucose uptake and fatty acid oxidation8,9 and decreases lipid accumulation in the tissues.10 In contrast to the effect of ALA to inhibit AMPK in the hypothalamus, ALA activated AMPK in skeletal muscle, resulting in enhanced fatty acid oxidation and reduced lipid accumulation.11 AMPK is expressed in vascular endothelial cells,12 and AMPK dysregulation has been suggested to contribute to endothelial dysfunction.10 We hypothesized that ALA may improve vascular function via activation of AMPK in vascular endothelial cells. In the present study, we examined whether endothelial dysfunction in obese Otsuka Long Evans Tokushima Fatty (OLETF) rats is associated with impaired AMPK activation in vascular endothelial cells and whether these defects can be normalized by ALA treatment.

Materials and Methods

For details regarding animal and cell experiments, measurement of various metabolic parameters, and markers for apoptosis and reactive oxygen species (ROS), please see the online supplement, available at http://atvb.ahajournals.org.
Animals
Four-week-old male OLETF rats and their lean controls, Long Evans Tokushima Otsuka (LETO) rats, were obtained from Tokushima Research Institute, Otsuka Pharmaceutical Co. (Tokushima, Japan). All experimental procedures were approved by the institutional animal care and use committee of the Asan Institute for Life Sciences.

Vascular Function Study
Endothelium-dependent and -independent vasorelaxations were measured using an isometric force displacement transducer (Hugo Sachs Elektronik KG D-7806) as described previously.13

Electron Microscopy and TUNEL Staining
Apoptosis in the aortic endothelium was examined by electron microscopy and TUNEL staining.

Cell Culture
Human aortic endothelial cells (HAECs) were obtained from Bio-Whittaker Inc. and maintained in the endothelial basal medium (BioWhittaker Inc.) supplemented with various growth factors and 2% FBS.

Western Blot Analysis of AMPK Phosphorylation and Isoform-Specific AMPK Activity
Phosphorylation and protein levels of AMPK were assayed by Western blot analysis using the antibodies directed against phosphopeptides based on the amino acid sequence surrounding Thr172 of the α-subunit of human AMPK and the antibody against α-subunit of human AMPK (Cell Signaling), respectively. For isoform-specific AMPK activity measurement, we immunoprecipitated lysates of human AMPK (Cell Signaling) against the g protein each) with specific antibodies (Upstate Biotechnology) against the 1- and 2-catalytic subunits of AMPK bound to protein G–Sepharose beads. Kinase activity was measured using synthetic “SAMS” peptide and [γ-32P]ATP as described.14

Quantification of Cell Apoptosis
Apoptosis was measured by ELISA assay (Boehringer Mannheim) and caspase 3, 8, and 9 activity assay using an Apo Alert caspase florescence assay kit (Clontech).

Adenoviral Gene Transfer of Dominant-Negative α1 and α2 AMPK
Plasmid encoding c-Myc–tagged forms of dominant-negative α1 and α2 AMPK were a kind gift from Dr J. Ha (Department of Molecular Biology, Kyung Hee University College of Medicine, Seoul, Korea). Adenoviruses containing β-galactosidase (Ad-β-gal) or mixture of dominant-negative α1 AMPK and α2 AMPK (Ad-DN-AMPK) were added to subconfluent HAECs at a concentration of 10 plaque-forming units (pfu) per cell for 1 hour at 37°C in DMEM without serum, as described previously.15

Statistical Analysis
All data are shown as mean±SEM. Comparisons between 2 groups were analyzed using unpaired Student’s t tests, and among multiple groups by ANOVA, followed by a post hoc analysis using the Tukey’s multiple comparison test (SPSS). A P value <0.05 was considered to be statistically significant.

Results
Metabolic Parameters
Compared with LETO rats, OLETF rats had higher body weight, blood pressure, and fasting plasma levels of triglyceride, free fatty acid (FFA), glucose, and insulin. In addition, plasma levels of oxidative stress markers (ie, 8-hydroxydeoxyguanosine and malondialdehyde) were higher and urinary NO excretion was lower in OLETF rats than in LETO rats (Table I, available online at http://atvb.ahajournals.org). ALA treatment completely normalized all of these changes in OLETF rats except for systolic blood pressure, which was substantially lowered but not to the level of LETO rats. These effects of ALA treatment seemed to be mediated by its effect to reduce food intake because pair feeding of OLETF rats resulted in exactly the same effects. Plasma adiponectin concentrations in OLETF rats were unaltered by ALA treatment.

Endothelium-Dependent Vascular Relaxation
Compared with LETO rats, OLETF rats showed reduced vasodilation of aortic rings in response to acetylcholine, indicating impaired endothelium-dependent vascular relaxation. The endothelium-dependent vasorelaxation in aortic rings of OLETF rats was substantially improved by ALA treatment (Figure 1A). Vascular relaxation in response to sodium nitroprusside, an NO donor, was not different among groups (Figure 1B), indicating that vascular reactivity to NO is intact in OLETF rats. Pair feeding also significantly improved endothelium-dependent vascular function in OLETF rats, but this improvement was significantly less than the improvement achieved by ALA treatment (Figure 1C).

NO Synthesis and Lipid Contents in Aortic Endothelium
To evaluate whether the changes in endothelium-dependent vasodilation were attributable to changes in NO synthesis, we measured acetylcholine-stimulated NO synthesis in isolated aortas. NO synthesis was significantly lower in aortas of OLETF rats compared with that of LETO rats (Figure 2A). NO synthesis was substantially increased in aortas of OLETF rats by ALA treatment. Pair feeding also significantly im-
proved NO synthesis, but this effect was smaller than that of ALA treatment.

Triglyceride and lipid hydroperoxide levels in aortic endothelial cells of OLETF rats were significantly higher than those in LETO rats (Figure 2B and 2C). These changes were reversed completely by ALA treatment but only partially by pair feeding.

**AMPK Phosphorylation and Activities in Aortic Endothelium**

AMPK phosphorylation was lower in aortic endothelium of OLETF rats compared with that of LETO rats, and ALA treatment completely normalized it (Figure 2D). AMPK activities were also lower in aortic endothelium of OLETF rats compared with those of LETO rats. ALA treatment increased \( \alpha_1 \) (Figure 2E) and \( \alpha_2 \) (Figure 1, available online at http://atvb.ahajournals.org) AMPK activities. Interestingly, pair feeding had no effect on these measurements despite its effects on blood metabolic parameters, which were identical to those of ALA treatment (Table I).

These data suggest that the effect of ALA treatment on AMPK phosphorylation and activities might be a direct effect of ALA, independent of the effects of ALA on body weight and metabolic parameters.

**Effects of acute administration of ALA on AMPK Activation and Endothelial Function**

To further demonstrate direct effects ALA on AMPK and vascular function, we administrated ALA to 15-week-old OLETF rats (n=12) by an intraperitoneal injection (75 mg/kg body weight). AMPK phosphorylation in aortic endothelium increased 2.3-fold 30 minutes after the ALA administration and remained elevated thereafter until 240 minutes (Figure 2F). Acute administration of ALA also improved endothelium-dependent vascular relaxation, increased NO production, and decreased lipid hydroperoxide levels in the isolated aorta (Figure II, available online at http://atvb.ahajournals.org).

**Endothelial Cell Apoptosis**

Many in vitro studies have suggested that endothelial cell apoptosis may be an important early event in the pathogenesis of atherosclerosis. Examination by electron microscopy revealed that lumenal walls of aortas of LETO rats were well covered with endothelial cells (Figure 3A). In contrast, in OLETF rats, multiple foci of endothelial cell loss were noticed on lumenal walls of the aorta, and the subendothelial matrix was exposed to the lumen (Figure 3B). Many of the endothelial cells showed apoptotic profiles with nuclear fragmentation and disconnection from neighboring endothelial cells and from underlying subendothelial matrix (Figure 3C). The ultrastructural characteristics of aortas of ALA-treated OLETF rats were between those of LETO rats and of OLETF rats. Intercellular gap junctions and interdigitations with surrounding tissue were relatively well preserved (Figure 3D). TUNEL staining (Figure 3E) revealed that the number of apoptotic endothelial cells was significantly higher in aortas of OLETF rats than in those of LETO rats, and that ALA treatment of OLETF rats substantially lowered it (Figure 3F). Pair feeding also lowered the number of apoptotic endothelial cells, but this effect was significantly smaller than the effect of ALA treatment (Figure 3F).

**AMPK and Apoptosis in cultured Endothelial Cells**

It was suggested that endothelial dysfunction in metabolic syndrome is mediated, at least in part, by elevated circulating FFA levels. Incubation of HAECs with 300 \( \mu \)mol/L linoleic acid led to a 2.5-fold increase in apoptosis (Figure 4A), which was accompanied by increases in caspase-3, caspase-9 (a measure of mitochondrial pathway), and caspase-8 (a measure of death receptor signaling pathway) activities (Figure 4B through 4D). The linoleic acid–induced increases in apoptosis and caspase activities were all inhibited by 0.5 mmol/L ALA. Linoleic acid decreased AMPK phosphorylation in HAECs, and ALA substantially reversed this decrease (Figure 4E). ALA increased AMPK phosphorylation rapidly (ie, within 30 minutes; Figure 4F) in HAECs as observed in vivo (Figure 2F).
Effects of Dominant-Negative α1 and α2 AMPK

Mixture of dominant-negative α1 and α2 AMPK nearly completely reversed the effects of ALA against the changes in intracellular triglyceride, ROS generation, and apoptosis induced by linoleic acid, indicating that these effects of ALA were mediated by AMPK activation (Figure 5A through 5D).

Effects of ALA on NAD(P)H Oxidase Activity and Mitochondrial Membrane Potential

Major sites of intracellular ROS generation are mitochondria and cell membrane NAD(P)H oxidase.18 The mitochondrial membrane potential increased rapidly and peaked at 30 minutes after exposure to linoleic acid compared with Ad-β-gal–treated control cells. The mitochondrial potential then decreased and became lower than that of the control cells. ALA treatment nearly completely prevented these changes in membrane potential. On the other hand, pretreatment with Ad-DN-AMPK substantially reversed the effects of ALA on mitochondrial membrane potential (Figure 6A). Similarly, linoleic acid increased NAD(P)H oxidase activity, and ALA decreased it. The effect of ALA on NAD(P)H oxidase activity was prevented by Ad-DN-AMPK (Figure 6B).

Discussion

Defective endothelium-dependent vascular relaxation is an early event in the development of atherosclerosis.19 We confirmed that endothelium-dependent vascular relaxation is impaired in obese OLETF rats20,21 and found that this impairment is associated with increased lipid accumulation and apoptosis and decreased NO synthesis and AMPK activities in endothelial cells. All of these alterations in endothelial cells and vascular dysfunction were substantially improved by ALA treatment.

Our previous study demonstrated strong anorexic effects of ALA in normal and obese rodents.7 In the present study, ALA reduced food intake (data not shown) and normalized body weight in OLETF rats. In addition, all of the metabolic changes (FFA, triglycerides, insulin, etc) observed in OLETF rats were normalized by ALA. The normalization of metabolic parameters seemed to be attributable to the reduction in food intake because it also occurred in pair-fed, untreated OLETF rats; the metabolic parameters were identical between ALA-treated and pair-fed OLETF rats. Pair feeding (or normalization of metabolic parameters) alone also improved
vascular function and reduced the alterations (lipid accumulation, apoptosis, NO synthesis, etc) in endothelial cells of OLETF rats. However, these effects were only about a half of the effects of ALA treatment, suggesting that ALA improved vascular dysfunction in OLETF rats in part by reducing food intake but also by exerting additional effects. In regard to this, it is of extreme interest that decreased AMPK activities in endothelial cells of OLETF rats was completely normalized by ALA but little affected by pair feeding. In addition, intraperitoneal injection of ALA increased AMPK phosphorylation in aortic endothelial cells within 30 minutes and improved vascular dysfunction in OLETF rats. These data suggest that ALA directly activates AMPK in endothelial cells and that this effect leads to the improvement of vascular dysfunction in OLETF rats beyond that with pair feeding or metabolic normalization. However, to prove the causal relationship between AMPK activation and improvement of vascular function, it would be necessary to demonstrate the effect of selective inactivation of AMPK in aortic endothelial cells, as was done in in vitro experiment.

In cultured HAECs, linoleic acid decreased AMPK phosphorylation and increased triglyceride accumulation, apoptosis, and ROS generation. ALA prevented the linoleic acid–induced decrease in AMPK phosphorylation, and this effect was associated with normalization of triglyceride level, apoptosis, and ROS generation in the presence of linoleic acid. Furthermore, expression of dominant-negative AMPK in these cells led to inhibition of ALA effects. Together, these data suggest that AMPK activity in endothelial cells is an important regulator of endothelial function.

A key feature of defective endothelium-dependent vasodilation is reduced bioavailability of NO.19 Consistent with previous studies in other animal models of obesity and diabetes,22 aortas isolated from OLETF rats displayed attenuated vascular responses to acetylcholine but not to the direct-acting exogenous NO donor sodium nitroprusside. Urinary NO excretion and acetylcholine-stimulated NO synthesis in aortic endothelium were significantly reduced in OLETF rats compared with LETO rats. These results suggest that vascular reactivity to NO is intact, but NO bioavailability is reduced in OLETF rats. NO bioavailability is determined as the balance between NO production and removal. Evidence from experimental animals with diabetes suggests that the most likely mechanism underlying reduced NO bioavailability is inactivation of NO by oxygen-derived free radicals.23 Bakker et al4 proposed that increased availability of lipid (ie, triglyceride and long chain fatty acyl coenzyme A [LCAC])

Figure 5. Effects of dominant-negative AMPK on linoleic acid–induced intracellular triglyceride accumulation (A), ROS generation (B), apoptosis (C), and caspase-3 activity (D) in HAECs. HAECs were infected with adenoviruses containing plasmids coding for either mixture of α1 and α2 dominant-negative AMPK (Ad-DN-AMPK) or β-galactosidase (Ad-β-gal; control). Two days after infection, cells were incubated for 16 hours in media containing 0.5% FBS and 300 μmol/L linoleic acid, with or without 0.5 mmol/L ALA. *P<0.01 vs Ad-β-gal; **P<0.01 vs linoleic acid; ***P<0.01 vs ALA.

Figure 6. Effects of ALA on mitochondrial membrane potential (Ψm; A) and NAD(P)H oxidase activity (B). In A, time course of changes in Ψm for 6 hours is shown. Ψm was assessed by loading HAECs with 50 nmol/L TMRM (tetramethylrhodamine methyl ester) for 20 minutes. The uptake of TMRM increases with increased Ψm. *P<0.01 vs ALA. LA indicates linoleic acid. In B, NAD(P)H oxidase activity was assessed by measuring NAD(P)H-induced production of superoxide in HAECs. Data are expressed as mean±SEM. *P<0.01 vs Ad-β-gal; **P<0.01 vs linoleic acid; ***P<0.01 vs ALA.
in vascular endothelial cells could cause oxidative stress by affecting the mitochondrial respiratory chain. Supporting this hypothesis, the present study demonstrates that intracellular contents of lipid (i.e., triglycerides) and the oxidative stress marker (i.e., lipid peroxide) were elevated in aortic endothelium of OLETF rats.

Oxidative stress has been also implicated to play a major role in cellular apoptosis. The present study demonstrates that the number of apoptotic cells was significantly increased in aortic endothelium of OLETF rats. In cultured HAECs, linoleic acid increased intracellular triglyceride, oxidative stress, and apoptosis. Thus, increased circulating levels of FFA and triglycerides in obese animals may increase oxidative stress and apoptosis in vascular endothelial cells. This may further contribute to the decrease in NO bioavailability and vascular dysfunction (Figure III, available online at http://atvb.ahajournals.org).

We are the first to show that AMPK phosphorylation was decreased in aortic endothelium of obese rats and to suggest that it could be causally related to vascular dysfunction in obese animals. The mechanism by which this change occurs is unclear. However, because AMPK functions as a fuel sensor in the cell and is activated when cellular energy is depleted, it is conceivable that surplus cellular energy in association with high plasma FFA or glucose levels is responsible for reduced AMPK activity in aortic endothelium of OLETF rats. Consistent with this idea, we demonstrated that incubation of HAECs with linoleic acid significantly decreased AMPK activation. Reduced AMPK activity would decrease fatty acid oxidation by activating acetyl CoA carboxylase and increasing intracellular malonyl coenzyme A levels. Thus, increased FFA flux from the circulation and reduced FFA oxidation in aortic endothelium could lead to excessive accumulation of triglyceride and LCAC. This may be an early event that leads to a cascade of increased ROS generation, increased apoptosis, and decreased NO bioavailability in endothelial cells and vascular dysfunction, as discussed above.

ALA is well known as an antioxidant. ALA directly scavenges free radicals, chelates transition metal ion (e.g., iron and copper), increases cytosolic glutathione and vitamin C levels, and prevents toxicities associated with their loss. In addition, the present study demonstrates that antioxidant action of ALA is, at least in part, mediated by its effect on AMPK. Major sites of intracellular ROS generation are mitochondria and cell membrane NAD(P)H oxidase. The mitochondrial respiratory chain generates ROS when the electrochemical gradient between the mitochondrial inner membrane is high and the rate of electron transport is limited. Consistent with this concept, recent studies from our group and others have shown that high glucose or linoleic acid leads to significant increases in mitochondrial membrane potential (hyperpolarization) and ROS generation. In the present study, ALA treatment nearly completely prevented hyperpolarization induced by linoleic acid, and pretreatment with Ad-DN-AMPK reversed the effect of ALA. As stated above, LCAC is known to impair the flow of electrons through the electron transfer chain, and the reduction of LCAC levels by AMPK activation would be responsible for improvement in electron transfer and reduction in ROS generation.

We also found that linoleic acid increases and ALA decreases NADPH oxidase activity. Again, DN-AMPK reversed the effect of ALA, implicating that this effect is AMPK mediated. The mechanism by which AMPK activation decreases NAD(P)H oxidase activity is presently unknown but may be attributable to its effect on the cellular NADH/NAD+ redox state. It can be assumed that linoleic acid–induced mitochondrial membrane hyperpolarization, which reflects impairment of mitochondrial electron transfer and respiration, would increase NAD(P)H oxidase activity by increasing the cytosolic NADH/NAD+ ratio. Conversely, normalization of mitochondrial membrane potential by AMPK activation would decrease NAD(P)H oxidase activity by reducing the cytoplasmic NADH/NAD+ ratio.

In conclusion, our results suggest that reduced AMPK activity in endothelial cells may play an important role in the genesis of vascular dysfunction in obese rats, and that ALA may improve vascular dysfunction in obese rats by activating AMPK in endothelial cells. This study provides a rationale for the therapeutic use of ALA for vascular dysfunction in obese subjects.

Acknowledgments

This study was supported by the National Research Laboratory grant from the Ministry of Science and Technology (M104000000000804J000000810) and grants from the Korea Ministry of Health and Welfare (02-PJ1-PG10-20708-0007) and the Asan Institute for Life Sciences, (01-122, 01-279, 05-006) Republic of Korea.

References


α-Lipoic Acid Prevents Endothelial Dysfunction in Obese Rats via Activation of AMP-Activated Protein Kinase

Woo Je Lee, In Kyu Lee, Hyoun Sik Kim, Yun Mi Kim, Eun Hee Koh, Jong Chul Won, Sung Min Han, Min-Seon Kim, Inho Jo, Goo Taeg Oh, In-Sun Park, Jang Hyun Youn, Seong-Wook Park, Ki-Up Lee and Joong-Yeol Park

Arterioscler Thromb Vasc Biol. 2005;25:2488-2494; originally published online October 13, 2005;
doi: 10.1161/01.ATV.0000190667.33224.4c

Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2005 American Heart Association, Inc. All rights reserved.
Print ISSN: 1079-5642. Online ISSN: 1524-4636

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://atvb.ahajournals.org/content/25/12/2488

Data Supplement (unedited) at:
http://atvb.ahajournals.org/content/suppl/2005/10/20/01.ATV.0000190667.33224.4c.DC1

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Arteriosclerosis, Thrombosis, and Vascular Biology can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Arteriosclerosis, Thrombosis, and Vascular Biology is online at:
http://atvb.ahajournals.org//subscriptions/
Online Supplemental Methods

Animals

From 12 weeks of age, OLETF rats were given standard rat chow with (n=18) or without (n=18) 0.5% racemic ALA (Sigma, St. Louis, MO, USA). As ALA decreased food intake, a third group of OLETF rats, the pair-fed group (n=18), was given the same amount of food as that consumed by the ALA group on the previous day. At 27 weeks of age, blood pressure was measured by the photoplethysmographic method (model MK-1000, Muromachi Kikai, Tokyo, Japan). Urine and blood were collected. The thoracic aorta was excised and used for vascular function studies and histological examination (n = 6 each). To identify the mechanisms underlying ALA-induced improvement in vascular function, 6 rats were taken from each group at 15 weeks of age, and the levels of triglyceride and lipid peroxide in aortic endothelium were measured. The aortas from the remaining six rats from each group were used for assays for nitric oxide (NO) synthesis and Western blotting for AMPK.

Measurement of urinary NO excretion

Rats were placed in metabolic cages for 24 h and urine collected in a container bearing antibiotics. Concentrations of NOx (NO\textsubscript{2}\textsuperscript{-} and NO\textsubscript{3}\textsuperscript{-}) in urine were determined by NO-specific
chemiluminescence using an Antek nitrate/nitrite reduction assembly and an NO analyzer (models 745 and 7020; Antek Instruments, Inc., Houston, TX, USA).²

**Determination of metabolic parameters**

After a 5 h fast, animals were anesthetized with an intraperitoneal injection of 25 mg/kg pentobarbital. Blood was collected from the inferior vena cava. Plasma glucose levels were measured using the glucose oxidase method (Beckman Instrument, Palo Alto, CA, USA), plasma insulin levels by radioimmunoassay (Linco, St. Charles, MO, USA), plasma triglyceride and free fatty acids (FFA) levels using enzymatic assay kits (Sigma and Eiken Chemical Co., respectively, Tokyo, Japan). Plasma adiponectin levels were measured using rat RIA kit (Linco). Plasma 8-hydroxydeoxyguanosine (8-OHdG) and malondialdehyde (MDA) were measured using competitive ELISA (OXIS health Products, Portland, OR, USA) and HPLC (Waters Co. model 2690, Milford, MA, USA), respectively.

**Measurement of NO synthesis from aortic tissues**

Immediately after blood collection, the thoracic aorta was excised and cleaned of fat and adherent tissues. An isolated open vascular ring was incubated for 10 min in a sampling tube with 1.0 ml Kreb’s solution that was oxygenated with 95% O₂-5% CO₂. NO production by
aortic tissues was measured using the ISO-NO Mark II machine (World Precision Instruments, Sarasota, FL, USA) as described previously,\textsuperscript{3} before and after a 5-min stimulation with acetylcholine ($10^{-5}$ mol/l).

**Preparation of aortic endothelium**

Excised aortas were cut longitudinally, and endothelial cells were collected with a plastic scraper.\textsuperscript{4} These preparations of aortic endothelium were analyzed for triglyceride and lipid peroxide levels and AMPK protein and phosphorylation levels and activity.

**Measurement of triglyceride and lipid peroxide**

Triglyceride contents of aortic endothelium were determined in duplicate using an enzymatic assay kit (Sigma). Lipid peroxide levels were measured as described previously.\textsuperscript{5}

**Electron microscopy**

Thoracic aorta segments were fixed with 2% glutaraldehyde (Merck, Darmstadt, Germany) in 0.1 mol/l sodium cacodylate at room temperature. They were stained en bloc in uranyl maleate for 1 h, post-fixed in 1% osmium tetroxide, dehydrated and embedded in Epon 812 (Fluka Chemie, Buchs, Switzerland). Thin sections were stained with uranyl acetate and lead citrate and
examined using a Philips 400 electron microscope (Andover, MA, USA).

**TUNEL staining**

A piece of thoracic aorta segment was fixed with 4% paraformaldehyde in 0.1 N phosphate buffer for 24 h. TUNEL staining was performed to locate DNA fragmentation using an Apop-Tag kit (Oncor, Gaithersburg, MD, USA) according to the supplier’s instruction.

**Linoleic acid supplementation of culture media**

Linoleic acid (Sigma) was dissolved in 0.1 N NaOH in a boiling water bath in order to provide a 3 M stock solution. Appropriate volumes of freshly prepared stock solution were added slowly and with continuous agitation to medium containing fatty acid-free bovine serum albumin (100 µmol/l).

**ELISA measurement of apoptosis**

Cells were incubated for 16 h in EBM/0.5% FBS prior to the addition of agents. The levels of cytosolic histone-bound DNA fragments were measured using a cell death ELISA kit (Boehringer Mannheim, Indianapolis, IN, USA).
**Measurement of ROS generation**

HAECs were incubated for 15 min with 10 µM/ml carboxydichlorodihydrofluorescein diacetate (DCFH$_2$-DA) (Sigma) at 37°C. Increases in DCFH$_2$ oxidation were measured using a FACS (FACSCalibur, Becton Dickinson, San Jose, CA, USA).^6

**Measurement of mitochondrial membrane potential**

The degree of polarization of the mitochondria was determined by loading with tetramethylrhodamine (TMRM; Molecular Probes, Eugene, OR, USA) as described previously.^7 Thirty min after treating cells with or without ALA, cells were seeded to 96-well culture plates and exposed to linoleic acid for the given time periods. Cells were incubated with 50 nmol/l TMRM for 20 min at 37°C and then rinsed with Hanks’ balanced salt solution (10 mmol/l HEPES, pH 7.4, 150 mmol/l NaCl, 5 mmol/l KCl, 1 mmol/l MgCl$_2$, 1.8 mmol/l CaCl$_2$). The plate was immediately placed in a microplate spectrofluorometer (SPECTRAmax GEM-INI-XS; Molecular Devices, Sunnyvale, CA, USA), and the absorbance of TMRM was determined by 485-nm excitation and 590-nm emission.

**Measurement of NAD(P)H oxidase activity**

HAECs were washed twice with PBS, then lysed with lysis buffer (50 mmol/l Tris–HCl, 150
mmoll NaCl, 1 mmol/l EDTA, 1% Triton X-100, 10% glycerol, 1 mmol/l PMSF, 1 mg/ml aprotinin, 1 mg/ml leupeptin) and incubated for 1 h on ice. The lysate was centrifuged at 12,000 g for 20 min and the supernatant was saved. Protein content was determined using the Bradford method (Bio-Rad, Hercules, CA, USA). NAD(P)H oxidase activity was measured by lucigenin chemiluminescence. This assay was performed in a Krebs/HEPES buffer with 25 µM lucigenin as the electron acceptor, and NADH/NADPH (100 µM) as substrates. The reaction was started by addition of 25 µg protein, and photon emission was measured every 15 sec for 10 min in a microtiterplate luminometer (GENios, TECAN Inc., Austria).
References


6. van Reyk DM, King NJ, Dinauer MC, Hunt NH. The intracellular oxidation of 2’,7’-


Online Supplemental Figures

Online Figure I. Effects of ALA on α2 AMPK activity in aortic endothelial cells.

Data are expressed as mean ± SEM (n=6 in each group). * $P < 0.01$ vs. LETO (i.e., control) group, ** $P < 0.01$ vs. OLETF group.
Online Figure II

A

Concentration of acetylcholine (log M)

Relaxation (%)

LETO
OLETF
OLETF + ALA

B

NO (pmol/mg dry wt of aorta)

LETO          OLETF    OLETF+ALA

*  **
Online Figure II. Effects of acute administration of ALA on vasorelaxation (A), NO synthesis (B), and lipid peroxide levels (C).

Endothelium-dependent vascular relaxation, NO production and lipid peroxide levels were measured 2 hours after intraperitoneal injection of ALA. In B, open and closed bars represent basal and acetylcholine-stimulated NO synthesis, respectively. Data are expressed as mean ± SEM (n = 6 per group). * P < 0.01 vs. LETO group, ** P < 0.01 vs. OLETF group.
Online Figure III. Suggested effects of oxidative stress and alpha-lipoic acid (ALA) on vascular function.

Accumulation of lipid metabolites in vascular endothelial cells increases oxidative stress.

Oxygen-derived free radicals decrease the bioavailability of NO by directly quenching it.

Oxidative stress may also decrease NO bioavailability by increasing endothelial apoptosis. ALA improves vascular function, at least in part, by activating AMPK and reducing accumulation of lipid metabolites in endothelial cells.
Online Table. Body weight, blood pressure and plasma and urine metabolic markers in LETO rats, OLETF rats and OLETF rats treated with ALA.

<table>
<thead>
<tr>
<th></th>
<th>LETO</th>
<th>OLETF</th>
<th>OLETF + ALA</th>
<th>Pair-fed OLETF</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight (g)</td>
<td>530 ± 25</td>
<td>650 ± 33*</td>
<td>525 ± 20†</td>
<td>560 ± 22†</td>
</tr>
<tr>
<td>SBP (mmHg)</td>
<td>118.5 ± 2.1</td>
<td>165.7 ± 1.5*</td>
<td>132.7 ± 5.8†‡</td>
<td>135.4 ± 6.0†‡</td>
</tr>
<tr>
<td>DBP (mmHg)</td>
<td>86.5 ± 13.4</td>
<td>100.1 ± 2.6*</td>
<td>85.0 ± 9.1†</td>
<td>88.0 ± 9.3†</td>
</tr>
<tr>
<td>Triglyceride (mg/dl)</td>
<td>0.78 ± 0.11</td>
<td>2.85 ± 0.42*</td>
<td>0.59 ± 0.09‡</td>
<td>0.61 ± 0.10‡</td>
</tr>
<tr>
<td>FFA (µmol/L)</td>
<td>535.7 ± 47.8</td>
<td>748.1 ± 80.0*</td>
<td>577.1 ± 38.9†</td>
<td>580.1 ± 39.5†</td>
</tr>
<tr>
<td>Glucose (mmol/L)</td>
<td>7.1 ± 0.2</td>
<td>7.8 ± 0.2*</td>
<td>7.1 ± 0.1†</td>
<td>7.2 ± 0.1†</td>
</tr>
<tr>
<td>Insulin (pmol/L)</td>
<td>64.6 ± 5.1</td>
<td>139.4 ± 5.1*</td>
<td>78.2 ± 15.3†</td>
<td>79.5 ± 16.1†</td>
</tr>
<tr>
<td>Adiponectin (µg/ml)</td>
<td>3.5 ± 0.3</td>
<td>2.9 ± 0.3</td>
<td>3.0 ± 0.3</td>
<td>3.1 ± 0.3</td>
</tr>
<tr>
<td>MDA (µmol/L)</td>
<td>1.06 ± 0.07</td>
<td>1.55 ± 0.08*</td>
<td>1.10 ± 0.11†</td>
<td>1.20 ± 0.15†</td>
</tr>
<tr>
<td>8-OHdG (ng/ml)</td>
<td>0.09 ± 0.01</td>
<td>0.32 ± 0.02*</td>
<td>0.18 ± 0.02‡</td>
<td>0.20 ± 0.02‡</td>
</tr>
<tr>
<td>Urinary NO excretion</td>
<td>1.52 ± 0.48</td>
<td>0.64 ± 0.01*</td>
<td>1.48 ± 0.46†</td>
<td>1.10 ± 0.41†</td>
</tr>
</tbody>
</table>

SBP; systolic blood pressure, DBP; diastolic blood pressure

* *P < 0.005 vs. LETO, †P < 0.05 vs. OLETF, ‡P < 0.05 vs. LETO