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$_2^-$ and NO$_3^-$) 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, 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. 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.

**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 GEMINI 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
mmol/l 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 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)

LETO
OLETF
OLETF + ALA

Relaxation (%)

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 OLET</th>
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<tbody>
<tr>
<td>Body weight (g)</td>
<td>530 ± 25</td>
<td>650 ± 33*</td>
<td>525 ± 20†</td>
<td>560 ± 22†</td>
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<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>
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<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>
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<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>
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<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>
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<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>
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<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>
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<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>
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<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>
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<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>
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<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>
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<td>(pmol/100g body weight/d)</td>
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SBP; systolic blood pressure, DBP; diastolic blood pressure

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