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RESEARCH PAPER

Effects of α-lipoic acid on endothelial function in aged diabetic and high-fat fed rats

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Background and purpose: This study was conducted to investigate the effects of α-lipoic acid (α-LA) on endothelial function in diabetic and high-fat fed animal models and elucidate the potential mechanism underlying the benefits of α-LA.
Experimental approach: Plasma metabolites reflecting glucose and lipid metabolism, endothelial function, urinary albumin excretion (UAE), plasma and aortic malondialdehyde (MDA) and urinary 8-hydroxydeoxyguanosine (8-OHdG) were assessed in non-diabetic controls (Wistar rats), untreated Goto-Kakizaki (GK) diabetic and high-fat fed GK rats (fed with atherogenic diet only, treated with α-LA and treated with vehicle, for 3 months). Vascular eNOS, nitrotyrosine, carbonyl groups and superoxide anion were also assessed in the different groups.
Key results: α-LA and soybean oil significantly reduced both total and non-HDL serum cholesterol and triglycerides induced by atherogenic diet. MDA, carbonyl groups, vascular superoxide and 8-OHdG levels were higher in GK and high-fat fed GK groups and fully reversed with α-LA treatment. High-fat fed GK diabetic rats showed significantly reduced endothelial function and increased UAE, effects ameliorated with α-LA. This endothelial dysfunction was associated with decreased NO production, decreased expression of eNOS and increased vascular superoxide production and nitrotyrosine expression.
Conclusions and implications: α-LA restores endothelial function and significantly improves systemic and local oxidative stress in high-fat fed GK diabetic rats. Improved endothelial function due to α-LA was at least partially attributed to recoupling of eNOS and increased NO bioavailability and represents a pharmacological approach to prevent major complications associated with type 2 diabetes.
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Keywords: type 2 diabetes; high-fat diet; α-lipoic acid; oxidative stress; endothelial function; eNOS; nitric oxide; atherosclerosis

Abbreviations: α-LA, (±)-α-lipoic acid; AD, atherogenic diet; DHE, dihydroethidium; eNOS, endothelial nitric oxide synthase; GK rats, Goto–Kakizaki rats; MDA, malondialdehyde; ROS, reactive oxygen species; SNP, sodium nitroprusside; SO, soybean oil

Introduction

Endothelial dysfunction is an early and crucial event in the pathogenesis of atherosclerosis and cardiovascular disease. It is characterized by a shift in the actions of the endothelium towards reduced vasodilation, a proinflammatory state, and prothrombic properties and is associated with several risk factors, such as hypercholesterolaemia, hypertension, diabetes and ageing (Schalkwijk and Stehouwer, 2005). The predisposition to atherosclerosis in diabetes appears to be the consequence of pathogenic dyslipidaemia in insulin-resistant states, which is characterized by hypertriglyceridaemia, as well as increased concentrations of low-density lipoprotein cholesterol and reduced levels of high-density lipoprotein (HDL) cholesterol (Goldberg, 2001).

Diabetes-associated vascular disease affects multiple vascular beds (Goraya *et al*., 2002) and displays a significant inflammatory component (Burke *et al*., 2004). The cellular and molecular mechanisms underlying diabetic vascular disease remain to be clarified. The pathogenesis of endothelial dysfunction in type 2 diabetes is multifactorial. Hyperglycaemia, insulin resistance, dyslipidaemia, hypertension and advanced glycation end products have all been implicated in the pathogenesis of accelerated arterial disease in patients with diabetes (Creager *et al*., 2003). The fact that experimentally induced hyperglycaemia and hyperinsuli-

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naemia can reduce endothelium-dependent vasodilation suggests that these metabolic parameters can have direct effects on the arterial wall (Arcaro *et al*., 2002). Reduced endothelium-derived nitric oxide (NO) is thought to contribute to endothelial dysfunction and atherosclerosis through the loss of the vasodilatory, anti-inflammatory and antiproliferative properties of NO, respectively (Cai and Harrison, 2000; Creager *et al*., 2003). NO availability is the result of a delicate balance between NO production via endothelial nitric oxide synthase (eNOS) and inactivation by reactive oxygen species (ROS), such as superoxide. Elevated blood glucose levels drive production of ROS via multiple pathways, resulting in uncoupling of mitochondrial oxidative phosphorylation and eNOS activity, reducing NO availability and generating further ROS (Cai and Harrison, 2000).

α-Lipoic acid (α-LA) is a naturally occurring compound that appears to be useful in treating pathologies associated with oxidative stress. For example, α-LA has been safely used for more than 30 years in Europe to prevent and treat complications associated with diabetes, such as protein glycation, abnormal glucose utilization, polyneuropathy and cataracts (Ziegler and Gries, 1997). α-LA can also bind to, or chelate, metals like iron and copper. Exogenously supplied α-LA is readily absorbed by cells and tissues and then rapidly reduced to its potent antioxidant form, dihydrolipoate (Packer *et al*., 2001).

The influence of α-LA on the development of diabetes-associated vascular changes is poorly understood. It has recently been reported that α-LA can reduce dyslipidaemia associated with obesity in rats (Lee *et al*., 2005) and with the development of diabetes in streptozotocin-treated/apolipoprotein E-deficient mice (Yi and Maeda, 2006). The aim of our study was to assess the effect of α-LA on development of dyslipidaemia, local and systemic oxidative stress and NO-related endothelial dysfunction in aged Goto–Kakizaki (GK) rats, a model of non-obese type 2 diabetes. We show here that α-LA leads to significant diminishing of all biomarkers of oxidative stress evaluated and significantly ameliorates the accelerated atherosclerosis, microalbuminuria and endothelial dysfunction observed in aged diabetic GK rats with hyperlipidaemia through a mechanism that involves eNOS recoupling.

Materials and methods

Animals

All animal procedures and care were in accordance with the Portuguese Law on Experimentation with Laboratory Animals (last amendment, 2004), which is based on the principles of laboratory animal care as adopted by the American Heart Association and the Declaration of Helsinki.

Adult male Wistar and spontaneously diabetic GK rats (15 months of age) were obtained from the local breeding colony in Coimbra (Portugal). Control animals were fed, *ad libitum*, with a standard commercial pellet chow (Diet AO4; Panlab, Barcelona, Spain) and allowed free access to water; they were kept in rooms with 12-h periods of light and darkness. The GK rats were divided into four groups. One group served as control, whereas the other three experimental groups of GK rats were fed with an atherogenic diet (AD) containing 70% AO4 standard Chow, 7.5% cocoa butter and 1.25% cholesterol for 3 months. (1) Control GK group with AD only; (2) GK/SO group in which rats received, 3 days per week during 3 months, an intraperitoneal injection of soybean oil (SO; $2\,ml\,kg^{-1}$ body weight, the vehicle of α-LA) and (3) GK/α-LA group in which rats received intraperitoneal (±) α-LA ($50\,mg\,kg^{-1}$ body weight) in SO, again 3 days per week for 3 months. Urine and blood were collected at the end of treatment. After 3 months, the animals were killed. The aorta was excised and used for vascular function studies and histological examination.

Determination of metabolic and oxidative stress parameters

After a 15-h fast, animals were anaesthetized with ketamine/chlorpromazine. Blood was taken by heart puncture for determination of lipids, lipid peroxides, free fatty acid levels and insulin. For glucose tolerance tests, rats were fasted overnight and were given an intraperitoneal injection of glucose ($1.75\,g\,kg^{-1}$ body weight) in phosphate-buffered saline. Blood glucose was determined by sampling from the tail vein at 0, 30, 60, 90 and 120 min after injection by a glucose-oxidase method using a glucometer (GlucometerElite; Bayer Portugal SA, Lisbon, Portugal) and compatible reactive test strips. Fasting plasma lipids (total and HDL cholesterol, triglycerides and phospholipids) and plasma insulin levels were quantified using commercially available kits and by an in-house enzyme-linked immunosorbent assay (Seiça *et al*., 2004), respectively. Plasma free fatty acid levels were evaluated using enzymatic assay kits (Roche Applied Science, Amadora, Portugal). Rats were placed in metabolic cages for 24 h and urine was collected. Urinary 8-hydroxydeoxyguanosine, albuminuria and plasma malondialdehyde (MDA) were measured using competitive enzyme-linked immunosorbent assay (OXIS Health Products, Portland, OR, USA and Nephrat II, Exocell, Philadelphia, PA, USA) and HPLC (Santos *et al*., 2001), respectively.

Isometric tension studies

The aorta was rapidly excised, freed of connective tissue and divided into two segments (4 mm width). Ring segments were mounted between stainless steel triangles into individual organ chambers filled with oxygenated (95% O_2 and 5% CO_2) Krebs–Henseleit buffer (37 °C, pH 7.4) (composition in mM: NaCl 119; KCl 4.7; $CaCl_2$ 1.6; $MgSO_4$ 1.2; $NaHCO_3$ 25; KH_2PO_4 1.2; glucose 11.0). Indomethacin at a concentration of 10 μM was present in some experiments to inhibit prostaglandin synthesis. Aortic rings were subject to a resting tension of 2 g. After equilibration for 60 min, all vessels were preconstricted with 0.3 μM phenylephrine. Ligand-stimulated receptor-mediated NO bioavailability was assessed by a dose-dependent relaxation to acetylcholine (ACh; 10 nM–1 mM), whereas sodium nitroprusside (SNP, from 10 nM to 1 mM) was used as an endothelium-independent agonist. Relaxation responses to ACh and SNP were expressed as percentage of relaxation from a submaximal phenylephrine-induced constriction (10^{-7} M). Other aortic rings were used

to test the contractile response to phenylephrine (from 1 nM to 10 μM). A cumulative dose–efficacy curve was determined. Regression analysis using three data points along the linear section of the concentration–response curve was applied to generate an equation from which the EC_{50} values were determined.

Detection of superoxide

Unfixed frozen, 30-μm-thick cross sections of proximal aorta were incubated with dihydroethidium (DHE; 2×10^{-6} M) in phosphate-buffered saline for 30 min at 37 °C in a humidified chamber protected from light. DHE is oxidized on reaction with O_2^- to ethidium bromide, which binds to DNA in the nucleus and fluoresces red (Miller *et al*., 1998). Polyethylene glycol-superoxide dismutase (500 U ml^{-1}) abolished ethidium bromide fluorescence, confirming specificity of the fluorescent signal for O_2^- (Supplementary Figure 1). For ethidium bromide detection, images were obtained with a Bio-Rad MRC-600 laser scanning confocal microscope equipped with a krypton/argon laser. Fluorescence was detected with a 568-nm filter. Normal and diabetic tissues were processed and imaged in parallel. Laser settings were identical for acquisition of images from normal and diabetic specimens.

Assessment of aortic atherosclerosis

The whole aortas were collected and stained with Oil Red O (60% solubilized in propylene glycol) to detect the lipids present in lesions, as described previously (Cayatte *et al*., 2001). The entire aortic wall was photographed and scanned digitally, and planimetry of Oil Red O-positive stained lesions was performed on the digitized images using Scion Image software.

Nitrite detection

Nitrite levels were determined as an index of NO generation in aortic homogenates by the Griess reaction after conversion of nitrate into nitrite by nitrate dehydrogenase (Green *et al*., 1982). An aliquot of the supernatant was mixed with an equal volume of Griess reagent (sulphanilamide, 1% w/v; naphthylethylenediamine dihydrochloride, 0.1% w/v and orthophosphoric acid, 25% v/v) and incubated at room temperature for 10 min. The absorbance of the samples at 540 nm was determined and compared with those of known concentrations of sodium nitrite. The amount of nitrite formed was normalized to the protein content of the respective aorta.

Aortic MDA content

Lipid peroxidation was assessed by measuring the content of MDA, one of the end products of lipid peroxidation in tissues. Aortic samples were homogenized in ice-cold 10% (w/v) trichloroacetic acid (100 mg of tissue per 5 ml of trichloroacetic acid). After centrifugation, a portion of the supernatant was added to an equal volume of 0.6% (v/v) thiobarbituric acid, and the mixture was heated at 100 °C for 20 min. The resulting MDA concentration was calculated from the absorption at 532 nm using a molar extinction coefficient of 1.49×10^{-5} and was expressed as nm per (mg tissue) $\times 10^{-7}$ (Cassini *et al*., 1986).

Protein-bound carbonyl determination

Evaluation of aorta protein oxidation was performed by a modification of the method described by Levine *et al*. (1990). Protein carbonyl formation was used as an indicator of oxidized proteins. Briefly, frozen aortas were homogenized at 4 °C in 10 mM (Tris(hydroxymethyl)aminomethane)-HCl (pH 7.6, 1:10, w/v) containing 1% Nonidet P-40, 0.1% SDS and complete protease inhibitor cocktail (Roche Applied Science). Following vigorous vortexing, the homogenates were centrifuged at 14 000 *g* for 10 min at 4 °C and aliquots of the supernatant were used for protein determination. For derivatization of carbonyl-containing proteins, equal amounts of proteins were mixed with equal volume of 10 mM 2,4-dinitrophenylhydrazine in 10% trifluoroacetic acid and incubated at room temperature for 15 min. The reaction was stopped by precipitation of proteins with 20% trichloroacetic acid. The resulting pellet was washed with ethyl acetate/ethanol (1:1) to remove free 2,4-dinitrophenylhydrazine. The pellet was then solubilized with Laemmli sample buffer. For western blots, samples were resolved by SDS-polyacrylamide gel electrophoresis (PAGE) using 12% gels and transferred to polyvinylidene fluoride (PVDF) membranes. The membranes were probed with antibody to dinitrophenylhydrazone derivatives (Dako, Hamburg, Germany). Levels of carbonyl were quantified by densitometric analysis of all bands of the blot.

Nitrotyrosine

Nitrotyrosine, a biomarker of peroxynitrite formation, was determined in the aorta by estimating the levels of protein nitration using immunochemical methods. Existing antibodies were removed from aortic homogenates by incubating first with protein A/G agarose, followed by an overnight incubation with a rabbit anti-nitrotyrosine antibody (Upstate Biotechnology, Lake Placid, NY, USA). Protein A/G agarose was added to precipitate the nitrotyrosine-complexed antibody. Proteins were separated on 8% denaturing polyacrylamide gels, and the resulting blots were incubated with mouse anti-nitrotyrosine antibody (Upstate Biotechnology). Detection of nitrotyrosine was achieved using alkaline-phosphatase secondary antibody. A duplicated blot of conditions in Figure 7a incubated with sodium dithionite prior to incubation with anti-nitrotyrosine antibody was performed (Supplementary Figure 2).

Western blot analysis for eNOS

Segments of endothelium-intact thoracic aortas were washed with cold phosphate-buffered saline and chilled in buffer containing (in mM) Tris-HCl 50, NaCl 150, EDTA 1, ethylene glycol tetraacetic acid 0.1, as well as Nonidet P-40 0.1%, SDS 0.1% and deoxycholate 0.5%. Phenylmethylsulphonyl fluoride (1 mM), aprotonin (10 μg ml^{-1}), leupeptin (10 μg ml^{-1})

Table 1 Body weight, and plasma and urine metabolic markers in Wistar and GK rats, and in GK rats fed with AD with or without α-LA and SO at the end of treatment

	Wistar (n = 20)	*GK (n = 20)*	*GK + AD (n = 10)*	*GK + AD + SO (n = 10)*	*GK + AD + LA (n = 10)*
Body weight gain (g)	2.4 ± 0.8	1.5 ± 0.7	7.6 ± 2.6	50.1 ± 6.4[a]	11.9 ± 3.7
FBG (mg dl^{-1})	75.6 ± 1.9	115.3 ± 5.6[b]	151.7 ± 7.9[c]	129.6 ± 4.5	122.3 ± 9.3[d]
BG 2 h after a load (mg dl^{-1})	116.3 ± 4.0	332.5 ± 47.7[b]	419.1 ± 28.5[b]	338.6 ± 23.4[b]	365.6 ± 9.5[b]
FFAs (mmol l^{-1})	0.54 ± 0.15	0.74 ± 0.1[e]	0.69 ± 0.09[f]	0.9 ± 0.12[e]	0.89 ± 0.06[b]
UAE (mg/24 h)	2.2 ± 0.4	29.2 ± 4.7[b]	35.7 ± 4.1[b]	30.9 ± 2.8[b]	17.8 ± 2.9[d,g]

Body weight gain, fasting blood glucose (FBG), blood glucose (BG) 2 h after a glucose load in the tolerance test, plasma free fatty acids (FFAs) and urinary albumin excretion (UAE). Values are mean ± s.e.
[a] $P<0.001$ vs all groups.
[b] $P<0.001$ vs Wistar rats.
[c] $P<0.001$ vs GK control group.
[d] $P<0.05$ vs GK + AD group.
[e] $P<0.05$ vs Wistar rats.
[f] $P<0.01$ vs Wistar rats.
[g] $P<0.05$ vs GK + AD + SO group.

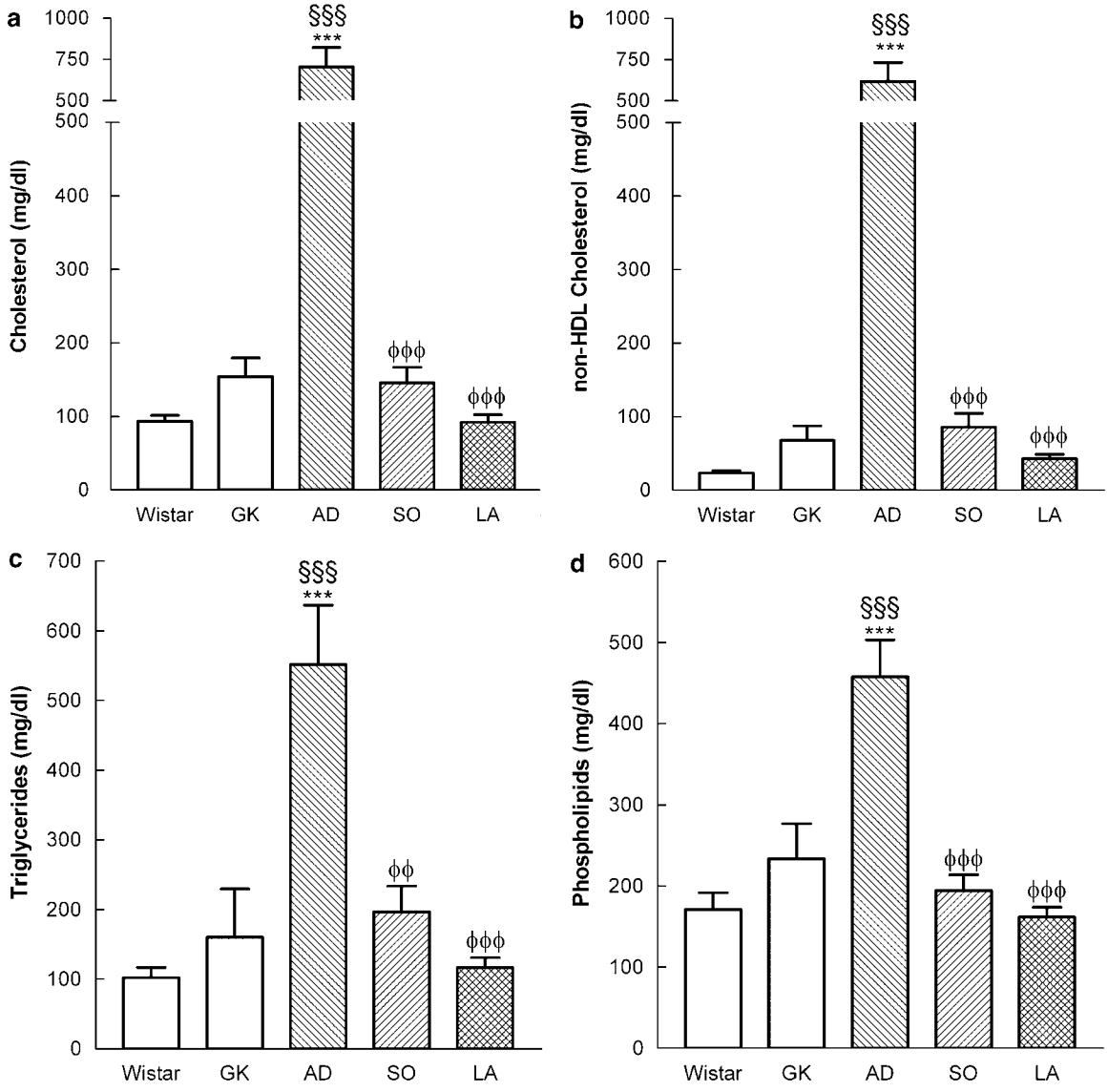

Figure 1 Effects of α-lipoic acid (α-LA) treatment on plasma lipid levels. Cholesterol (**a**), non-HDL cholesterol (**b**), triglycerides (**c**) and phospholipids (**d**) of Wistar and diabetic Goto–Kakizaki (GK) rats fed with standard chow (GK) or atherogenic diet (AD) with or without vehicle (soybean oil, SO) or α-LA. Data are expressed as mean ± s.e. ($n = 10$ animals in each group). In (**a–d**), ***$P<0.001$ vs Wistar group; §§§$P<0.001$ vs GK group; ϕϕ$P<0.01$, ϕϕϕ$P<0.001$ vs GK + AD group.

and pepstatin ($10\,\mu g\,ml^{-1}$), all from Sigma Chemicals (St Louis, MO, USA), were added as the protease inhibitors. Tissues were homogenized in a standard fashion, followed by centrifugation at $14\,000\,g$ for 20 min at 4 °C. The supernatants were collected and total protein concentration was determined. Samples containing 50 μg of protein were loaded onto an 8% SDS–PAGE gel, run and electroblotted onto nitrocellulose membrane. Prestained molecular weight marker proteins were used as standards for the SDS–PAGE. A Ponceau staining was performed to verify the quality of the transfer and to ensure equal protein loading. Blots were blocked in 5% skimmed non-fat milk in phosphate-buffered saline for 1 h, treated overnight with antibody against eNOS (Transduction Laboratory, Madrid, Spain) and then incubated with alkaline phosphatase secondary antibodies for 1 h. Immunoblots were developed with an ECF western blotting detection system (Amersham Biosciences, Carnaxide, Portugal).

Protein

Protein content was determined using a Bio-Rad protein assay kit.

Statistical analysis

All data were analysed by standard computer programs (GraphPad Prism PC Software version 3.0, analysis of variance) and are expressed as mean ± s.e. Significant

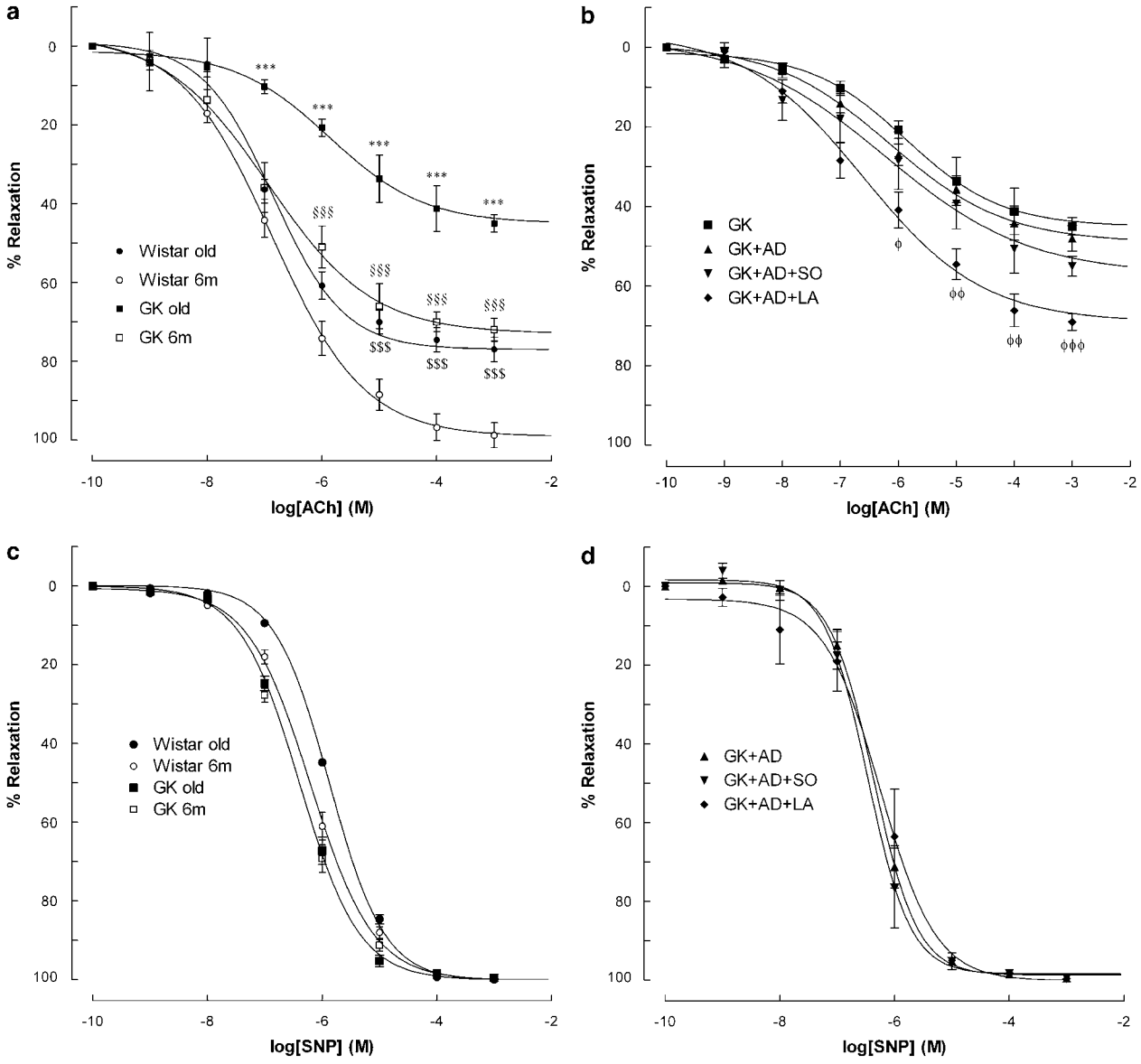

Figure 2 Effects of ageing and α-lipoic acid (α-LA) treatment on vasodilatory responses to acetylcholine (ACh) (**a** and **b**) and sodium nitroprusside (SNP) (**c** and **d**) in Wistar and Goto–Kakizaki (GK) rats after phenylephrine preconstriction of aortic segments. Vasorelaxation was measured using an isometric force displacement transducer. Data are expressed as mean ± s.e. ($n = 7$, 21 vascular ring preparations in 7 animals per group). Rats are 18 months old. In (**a**–**d**), ***$P<0.001$ vs Wistar 18 month group; $^{§§§}P<0.001$ vs GK group; $^{\$\$\$}P<0.001$ vs Wistar 6 month group; $^{\Phi}P<0.05$, $^{\Phi\Phi}P<0.01$, $^{\Phi\Phi\Phi}P<0.001$ vs GK + AD group.

Table 2 Maximal relaxation responses (%) and −log EC_{50} in isolated aorta arteries of 18-month-old variously treated spontaneously diabetic Goto–Kakizaki (GK) rats and age-matched non-diabetic Wistar rats

	Wistar	*GK*	*GK + AD*	*GK + AD + SO*	*GK + AD + LA*
Acetylcholine					
EC_{50}	6.85 ± 0.1	5.87 ± 0.07[a]	6.16 ± 0.07[b,c]	6.23 ± 0.23[b]	6.6 ± 0.1[d]
Maximal relaxation (%)	78.5 ± 3.1	45.0 ± 2.2[a]	49.0 ± 3.2[a]	55.0 ± 2.5[a]	70.0 ± 2.1[e,f]
Sodium nitroprusside					
EC_{50}	5.87 ± 0.04	6.4 ± 0.03[a]	6.4 ± 0.015[a]	6.47 ± 0.03[a]	6.25 ± 0.07[a]
Maximal relaxation (%)	99.9 ± 0.1	99.6 ± 0.01	99.5 ± 0.15	99.5 ± 0.11	99.5 ± 0.05

Values are mean ± s.e. ($n = 7$; 21 vascular ring preparations in seven animals per group). EC_{50} values are presented as the negative logarithm ($-\log_{10} EC_{50}$) of concentration of the agonist.
[a]$P < 0.001$.
[b]$P < 0.01$ vs Wistar rats.
[c]$P < 0.01$ vs GK control group.
[d]$P < 0.05$.
[e]$P < 0.001$ vs GK + AD group.
[f]$P < 0.01$ vs GK + AD + SO group.

differences were evaluated using either the *t*-test or analysis of variance. $P < 0.05$ was considered significant. Dose–response curves were fitted by nonlinear regression with simplex algorithm. Relaxation responses were given as the percentage of phenylephrine preconstriction. Comparisons of dose–response curves were evaluated by two-way analysis of variance for repeated measures.

Reagents

(±)-α-Lipoic acid (DL-6,8-thioctic acid, T-5625) and SO (S-7381) were from Sigma Chemicals; DHE was from Invitrogen-Molecular Probes (Barcelona, Spain) and the plasma free fatty acid enzymatic assay kit was from Roche Applied Science. All other reagents were from Sigma or Merck (Darmstadt, Germany) where not specified.

Results

Animal characteristics

Food consumption and water intake did not significantly change over the experimental period among the different groups studied (data not shown). GK rats (15 months old) destined to be treated with vehicle or α-LA were initially matched with regard to plasma levels of glucose, insulin and plasma free fatty acids. These indices are commonly used as indicators of the severity of the diabetic state. Indeed, most of the rats used in our experiments exhibited, in the beginning of the study, similar fasting plasma glucose levels. Physical and metabolic parameters concerning the above animals revealed that the gain in body weight among the three different groups, including control, GK and α-LA-treated GK rats was similar during the 3-month experimental period, the exception being the GK group treated with SO (Table 1). Fasting and 2 h after load, glucose levels and plasma free fatty acids during the post-absorptive state were elevated in GK rats when compared to their corresponding control Wistar values. Treatment with α-LA for 3 months effectively reduced circulating concentrations of fasting glucose, an effect also observed with SO treatment (Table 1). Urinary albumin excretion was significantly higher in GK rats compared to age-matched Wistar rats. Feeding the AD diet for 3 months to GK rats did not further increase urinary albumin excretion, and supplementation with α-LA significantly reduced it (Table 1).

Although GK rats exhibited normal lipid profile, high-fat-fed GK rats had elevated levels of triglycerides, total and non-HDL cholesterol and phospholipids when compared to control diabetic rats (Figure 1). The plasma levels of cholesterol, triglycerides, phospholipids and non-HDL cholesterol were completely brought down to normal in the GK groups treated with SO and with α-LA.

NO-dependent vascular relaxation in rat aorta

Ageing progressively impairs endothelium-dependent vasodilation in aorta arterial rings. Indeed, maximal endothelium-mediated relaxation of phenylephrine-precontracted rings in response to ACh declined by 23 and 55% for Wistar and GK rats, respectively (Figure 2a). In 6-month-old GK rats, endothelium-mediated vascular relaxation of phenylephrine-precontracted aorta arterial rings in response to ACh was impaired compared with age-matched Wistar rats, but the endothelium-independent relaxations to SNP were similar in both strains (Figures 2c and d). Preincubation of the arterial rings with the NOS inhibitor N^{ω}-nitro-L-arginine methyl ester HCl and the cyclooxygenase inhibitor indomethacin almost completely abolished relaxation by ACh in GK and Wistar rats (Supplementary Figure 3). The residual component due to other vasodilators is around 15% in our experimental conditions (18-month-old male rats). This residual response is expected in large conducting vessels such as aorta. The AD diet did not further impair vascular relaxation in response to ACh in aged GK rats. α-LA treatment (but not the SO vehicle) improved endothelium-dependent vascular relaxation during the AD diet (Figure 2b), but had no effect on endothelium-independent vascular relaxation responses to SNP (Figure 2d). No differences in maximal relaxation were observed in the concentration–effect curves for SNP between any of the groups of rats. Vascular sensitivity to SNP is decreased in Wistar rats, probably due to increased arterial stiffness. Endothelium-denuded rings from all groups showed a

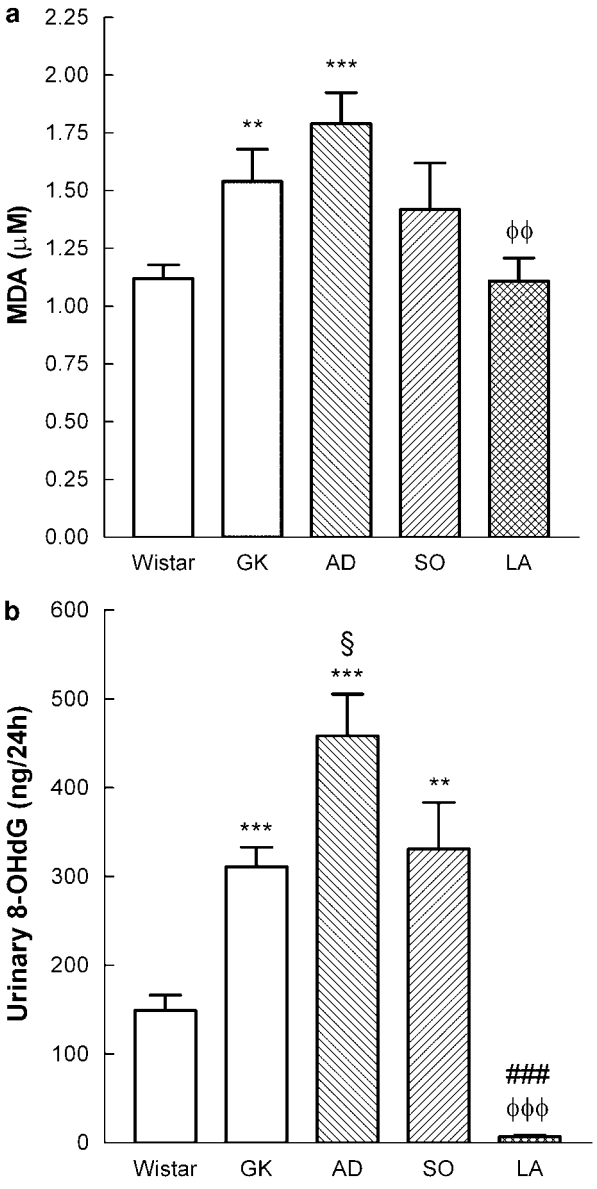

Figure 3 Reduction of oxidative stress parameters by α-lipoic acid (α-LA). Plasma malondialdehyde (MDA) (**a**) and urinary 8-hydroxydeoxyguanosine (**b**) levels in Wistar, Goto–Kakizaki (GK) control and GK rats fed with high-fat diet with or without vehicle (soybean oil, SO) or α-LA with 18 months old. Results are mean ± s.e. ($n = 10$ animals in each group). In (**a**) and (**b**), $^{**}P<0.01$, $^{***}P<0.001$ vs Wistar group; $^{\S}P<0.05$ vs GK group; $^{\phi\phi}P<0.01$, $^{\phi\phi\phi}P<0.001$ vs GK + AD group; $^{\#\#\#}P<0.001$ vs GK + SO.

similar relaxation to SNP, whereas no relaxation to ACh was observed. Detailed data on maximal relaxations and EC_{50} values are summarized in Table 2. These results indicated that treatment with α-LA improved this index of endothelial function in aged GK rats.

Oxidative stress biomarkers

Plasma levels of MDA and urinary levels of 8-hydroxydeoxyguanosine were significantly higher in GK rats when compared to age-matched Wistar rats (Figure 3). Feeding AD diet for 3 months to GK rats further increased these values by 20 and 47%, respectively (Figures 3a and b). Supplementation with α-LA reduced these oxidative stress parameters to basal levels (Figures 3a and b).

Diabetes-associated alterations in the eNOS enzyme system

Incubation of aortic rings in the presence of ACh (10 μM) increased the levels of NO metabolites (Figure 4a, right bar). Unstimulated values (Figure 4a, left bars) in the different groups did not differ. NO metabolites in aortic tissue of GK rats were reduced when compared with corresponding Wistar control values (Figure 4a). As NO in vascular endothelial cells is synthesized primarily by eNOS, we examined the possibility that a diabetes-related decrease in NO bioavailability might be due to a change in the expression of the NO-producing enzyme. Our data revealed that the expression of eNOS was decreased as a function of diabetes (Figures 4b and c). Densitometric analysis of the bands revealed that eNOS expression decreased by 25% in GK rats. There was a further increase with the AD diet, accompanied by a concomitant decrement in NO metabolites in aortic tissue of the high-fat-fed GK rats. Supplementation with α-LA (but not SO) completely restored NO output (Figure 4a) while partially restoring the eNOS expression in the aortic tissue (Figures 4b and c).

Diabetes-dependent elevation in vascular superoxide formation

Superoxide anion (O_2^-) constitutes the main oxidant of NO (Gryglewski *et al*., 1986). Accordingly, the premise that a diabetes-related decrease in NO bioavailability stems from an increase in O_2^- levels leads us to investigate *in situ* production of superoxide in the thoracic aorta. As illustrated by representative images and quantitative analysis, there was a threefold increase in superoxide production in diabetic aorta compared with control vessels ($P<0.05$; Figures 5a, b and e). The density of DHE staining was twofold higher in the aorta of diabetic rats fed with high-fat diet compared to GK control rats ($P<0.05$; Figures 5c and e). α-LA significantly reduced superoxide production in diabetic GK rats ($P<0.001$; Figures 5c–e). N^{ω}-Nitro-L-arginine methyl ester HCl (100 μM) significantly reduced superoxide production in diabetic GK rats fed with high-fat diet (Supplementary Figure 1b).

MDA, protein-bound carbonyl and nitrotyrosine in aortic vessels

Further experimentation was conducted to investigate the hypothesis that the state of oxidative stress is heightened in diabetic aortic vessels. Aortic contents of MDA and protein-bound carbonyls in GK rats were elevated by 85 and 30%, respectively, when compared to corresponding control values (Figures 6a–c). MDA and protein-bound carbonyl levels are sensitive indicators for lipid peroxidation and protein oxidation, respectively (Dalle-Donne *et al*., 2003; Del Rio *et al*., 2005). These indicators were further increased with high-fat diet by 107 and 77%, respectively, when compared to GK control group (Figures 6a–c). The MDA content of the aortic vessel wall was significantly reduced to control levels after treatment with α-LA, while levels of protein-bound carbonyls were also significantly decreased (Figures 6a–c).

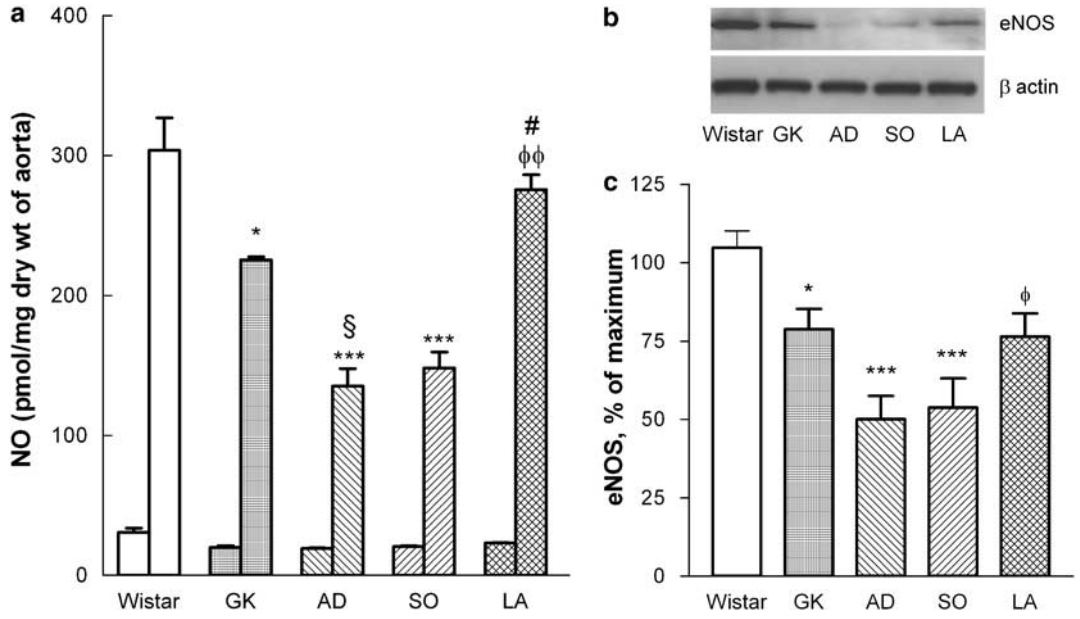

Figure 4 Effects of α-lipoic acid (α-LA) on aortic NO bioavailability and endothelial nitric oxide synthase (eNOS) expression in aortic tissue. (**a**) NO metabolites were assessed in aortic homogenates using the Griess reaction. In each group, left and right bars represent basal and acetylcholine (ACh)-stimulated NO synthesis, respectively. (**b**) Representative western blot analysis of eNOS protein expression in aortas of Wistar, Goto–Kakizaki (GK) control and GK rats fed with high-fat diet with or without vehicle (soybean oil, SO) or LA; 30 μg of protein from aortic lysates was resolved by SDS–polyacrylamide gel electrophoresis (PAGE), transferred to nitrocellulose membrane and probed with anti-eNOS antibody as described in Materials and methods. (**c**) Averaged densitometry data for diabetic group expressed as a percentage of elevation over the control value established as 100% previously normalized with β-actin values. Data are expressed as mean ± s.e. ($n=7$ animals per group). In (**a–c**), $^{*}P<0.05$, $^{***}P<0.001$ vs Wistar group; $^{\S}P<0.05$ vs GK group; $^{\phi}P<0.05$, $^{\phi\phi}P<0.01$ vs GK + AD group; $^{\#}P<0.05$ vs GK + SO group.

Accordingly, we sought to investigate whether the decrease in eNOS expression, together with enhanced O_2^- production in aortic tissue of GK rats, was associated with peroxynitrite formation and the nitration of tyrosine residues. A western blot analysis of aortic proteins revealed that the immunoreactive nitrotyrosine levels (an index of peroxynitrite formation *in vivo*) were elevated in GK rats compared with the Wistar counterpart (Figures 7a and b, and Supplementary Figure 5). The data are consistent with the concept that the overproduction of superoxide during diabetes results in the formation of peroxynitrite at the expense of NO. The latter oxidant is merely responsible for the nitration of aortic proteins. Similar to the other parameters, we observed an aggravation with high-fat diet that was decreased by the antioxidant α-LA.

Atherosclerotic lesions

Next, we evaluated how supplementation with α-LA affects atherosclerotic lesion development in 18-month-old GK rats (Figures 8a–d). Oil Red O staining showed the presence of intracellular lipid droplet accumulation only in the intima layer of GK arteries, while these lesions extended to the other layers in the GK rats fed with AD diet (76% of positive sections in hyperlipidaemic arteries). The atherosclerotic lesions both in long-term diabetes vs Wistar control and high-fat-fed GK vs GK control rats were significantly increased (Figures 8a–d and Supplementary Figure 4). Supplementation with α-LA prevented the enhancement of atherosclerosis that was induced by high-fat diet in GK rats (Figure 8d).

Discussion

The data from the present study demonstrate a novel role for α-LA in diabetes. Specifically, this treatment improved endothelial dysfunction by restoring NO signalling and abolishing eNOS-derived ROS production in aorta. Our observations are consistent with the earlier study by Hink *et al*. (2001), in which the lucigenin chemiluminescent signal of O_2^- was first found to be sensitive to N^{ω}-nitro-L-arginine methyl ester HCl in diabetes. The important novel findings are that α-LA was effective in partially restoring eNOS expression from its uncoupled state, and was also able to improve endothelial function and microalbuminuria in GK rats with hyperlipidaemia.

The GK rat is a non-obese spontaneous model of type 2 diabetes with mild hyperglycaemia, hyperinsulinaemia and insulin resistance. It has been previously shown that these animal models exhibit endothelial dysfunction as early as 4 months of age (Cheng *et al*., 2001). Feeding a high-fat, cholesterol-containing diet (AD) to GK rats resulted in a type 2 diabetes phenotype characterized by an increase in fasting hyperglycaemia, plasma triglycerides and cholesterol, and no changes in circulating free fatty acids, adipose mass and hyperinsulinaemia. Hyperlipidaemia and diabetes have been previously shown to induce endothelial dysfunction and increase neointimal formation in human and animal models

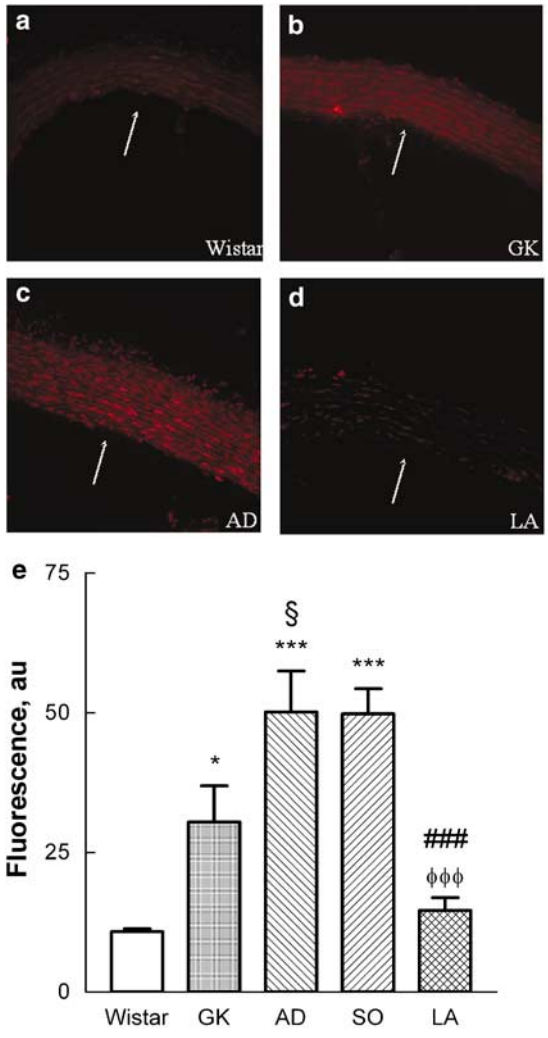

Figure 5 *In situ* detection of superoxide in rat aorta. Representative dihydroethidium (DHE)-stained aorta artery sections reflect O_2^- production with the different treatments. Arrows point to the endothelium. At identical laser and photomultiplier settings, fluorescence in diabetic Goto–Kakizaki (GK) and GK atherosclerotic vessel (**b** and **c**, respectively) was markedly increased compared with normal vessel (Wistar, **a**). Note the increased fluorescence reflecting O_2^- levels in the endothelium, intima and media (M) of GK aorta. DHE fluorescence decreased to basal levels in the GK + α-LA-treated group (**d**). Panel e contains quantification of the fluorescent ethidium signal in the different groups of arteries. Results are mean ± s.e. ($n = 10$ animals in each group). $^*P < 0.05$, $^{***}P < 0.001$ vs Wistar group; $^{\S}P < 0.05$ vs GK group; $^{\phi\phi\phi}P < 0.001$ vs GK + AD group; $^{\#\#\#}P < 0.05$ vs GK + SO group.

(De Vriese *et al*., 2000; Reis *et al*., 2000). In the current study, long-term hyperglycaemia and hyperinsulinaemia in this animal model of aged type 2 diabetes lead to severe endothelial dysfunction and microalbuminuria that was not exacerbated by hyperlipidaemia induced with the AD diet. It has recently been suggested that diabetes without associated dyslipidaemia accelerates atherosclerosis by mechanisms that are also activated by hyperlipidaemia (Kanter *et al*., 2007). In contrast, diabetes alone does not increase vascular disease in a vast number of animal models with hyperlipidaemia (Goldberg and Dansky, 2006). Thus, if the underlying mechanisms that accelerate atherosclerosis seem to be common, there is no further impairment in endothelial dysfunction and microalbuminuria with hyperlipidaemia.

The underlying cellular and molecular mechanisms associated with aged diabetes-related endothelial dysfunction were explored in the context of a number of possibilities. These include changes in the NO bioavailability and in the expression of eNOS, increased breakdown of NO due to augmented production of O_2^- and an imbalance in the rate of reactive oxygen/nitrogen species production and disposal within the microenvironment of the vessels. In this connection, we have demonstrated a marked reduction in aortic NO metabolites during diabetes and diabetes with hyperlipidaemia. This phenomenon appears to be due to a defect in NO-producing enzymes since the level of eNOS was downregulated in GK vascular tissues (control and with high-fat diet induced hyperlipidaemia) compared with their corresponding Wistar control values. Other authors, in agreement with this work, have previously shown that a prolonged diabetic state leads to downregulation of eNOS (Nagareddy *et al*., 2005). A decreased eNOS expression has also been reported in diabetic mice (Brownlee, 2001). The observation of diabetes-induced downregulation of eNOS combined with a decrease in vascular NO bioavailability led us to measure O_2^- anion, a well-known factor in the degradation of NO and its conversion into peroxynitrite (Gryglewski *et al*., 1986). We found that the level of this free radical was elevated in the aortic segments of the GK and GK + AD rats. In the current study, the potential mechanism of reduced eNOS function was explored to explain the reduced endothelium-dependent vasodilation in the model of type 2 diabetes with high-fat-diet-induced hyperlipidaemia. Enhanced oxidative stress in the vascular wall of diabetic rats led to diminished eNOS expression and reduced NO production. N^{ω}-nitro-L-arginine methyl ester HCl (100 μM) almost completely attenuated aortic O_2^- in the diabetic high-fat-fed rats, strongly suggesting uncoupling of eNOS (Supplementary Figure 1b). Uncoupling of eNOS by ROS has been described as an additional mechanism by which glucose contributes to endothelial dysfunction (Zou *et al*., 2002). Hyperglycaemia increases superoxide generation in endothelium by several different pathways (Molnar *et al*., 2005), and superoxide is known to inactivate NO and produce peroxynitrite (Cai and Harrison, 2000), which in turn, rapidly oxidizes the eNOS cofactor tetrahydrobiopterin to its inactive form and leads to uncoupling of eNOS (Alp and Channon, 2004). Hyperglycaemia has been shown to inhibit eNOS activity by phosphorylation by Akt. In addition, the lack of insulin in diabetes may contribute to the effect of decreased eNOS. Defective insulin signalling or insulin resistance in the arterial wall may lead to reductions of Akt-mediated activation of eNOS (Du *et al*., 2001; Kobayashi *et al*., 2004).

Impaired endothelium-dependent relaxation has been demonstrated in various vascular beds in different animal models of diabetes. It has been suggested that the excessive elevations in plasma glucose, insulin, low-density lipoprotein cholesterol, endothelin-1, angiotensin II and ROS that occur in diabetes are involved in the development of this dysfunction in several blood vessels (De Vriese *et al*., 2000). Several antioxidants have been proved to be beneficial by decreasing oxidative stress in improving endothelium-

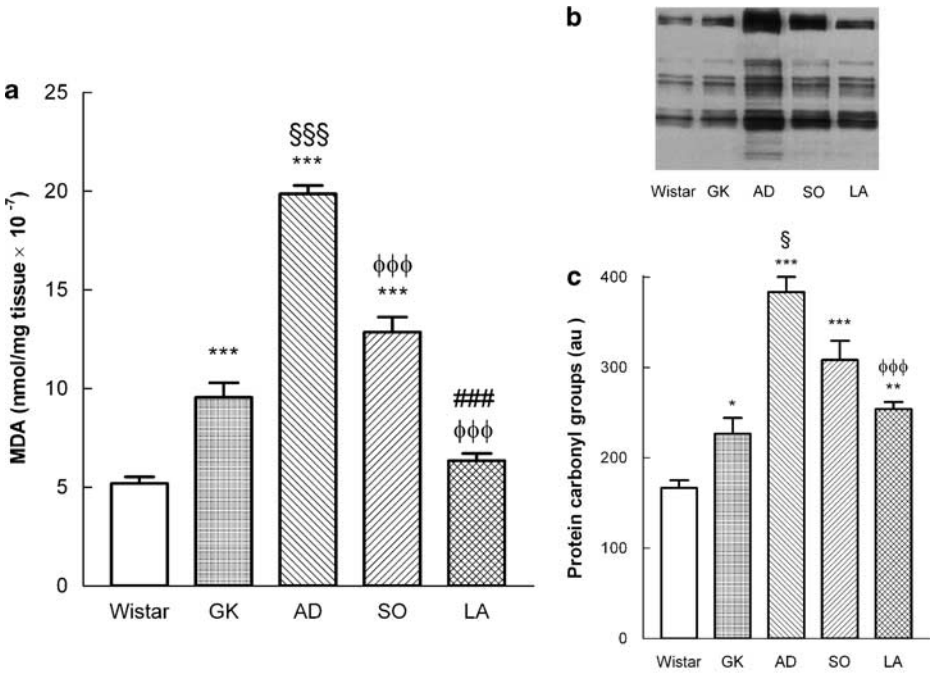

Figure 6 Effects of α-lipoic acid (α-LA) on aortic contents of malondialdehyde (MDA) (**a**) and protein-bound carbonyls (**b** and **c**) during diabetes. Markers of oxidative stress including MDA and protein-bound carbonyls were measured in aortic homogenates according to the procedures described in Materials and methods. Results are mean ± s.e. ($n = 10$ animals in each group). In (**a–c**), $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ vs Wistar group; $^{\S}P < 0.05$, $^{\S\S\S}P < 0.001$ vs GK group; $^{\phi\phi\phi}P < 0.001$ vs GK + AD group; $^{\#\#\#}P < 0.001$ vs GK + SO group.

dependent vascular relaxation (Da Ros *et al*., 2005). The potent antioxidant capacity of α-LA, which reduces oxidative stress both at systemic and local levels, was responsible for its beneficial action on atherosclerosis and vascular relaxation.

It has been previously shown that constituents of SO, such as α-linolenic acid, are capable of reducing cholesterol in hypercholesterolaemic patients (Lichtenstein *et al*., 2006). SO, the vehicle of α-LA in this study, had a significant effect in lipid profile with a significant reduction of plasma triglycerides, cholesterol and phospholipid levels, while no significant change was observed with respect to the oxidative stress parameters evaluated and on endothelium-dependent vascular relaxation. A reduction in circulating levels of cholesterol and triglycerides alone did not ameliorate endothelial dysfunction in this model of aged type 2 diabetes. In agreement with this work, several studies have reported a lack of effect of SO in endothelial function in humans (de Roos *et al*., 2002).

Kim *et al*. (2004) have recently reported that α-LA, a cofactor in the pyruvate dehydrogenase complex, exerts certain actions that mimic those of leptin, including reduced food intake and increased energy expenditure. α-LA, which is widely available as a powerful antioxidant, has been shown to protect against oxidative injury in various disease processes, including hypertension (Vasdev *et al*., 2005), diabetes (Song *et al*., 2005) and ischaemia reperfusion (Muller *et al*., 2003). α-LA has been previously demonstrated to inhibit endothelial dysfunction in other models of diabetes (Kocak *et al*., 2000; Lee *et al*., 2005). In addition, α-LA was able to upregulate eNOS expression in this study. In agreement with our work, it has been found that α-LA increases eNOS activity in human aortic endothelial cells (Visioli *et al*., 2002). It has also been shown that α-LA activates AMP-activated protein kinase in endothelial cells (Lee *et al*., 2005), an effect that was found to increase eNOS and NO production (Murakami *et al*., 2006). Another probable mechanism of action of α-LA involves activation of mitogen-activated protein kinase, which in turn will upregulate eNOS and increase NO production (Anter *et al*., 2004). It has recently been shown that asymmetric dimethylarginine plasma levels were significantly attenuated by α-LA in patients with end-stage renal disease (Chang *et al*., 2007). Asymmetric dimethylarginine is a known endogenous inhibitor of NOS and increased levels have been associated with ageing, type 2 diabetes and endothelial function. Modification of the redox status of thiol-containing proteins and attenuation of asymmetric dimethylarginine levels are possible mechanisms of action of α-LA.

In this study, the antiatherosclerotic effects of α-LA are not only due to a hypolipidaemic action, since SO has no effect on endothelial dysfunction and has also a lipid-lowering action. GK rats exhibited a severe impairment in endothelial-dependent vasodilation, partially due to ageing, and observed in age-matched Wistar control rats and also described in other animal models (Payne *et al*., 2003). Vascular sensitivity to SNP is increased in GK rats. Ageing and diabetes are known to increase chronic hypoxia and the expression of inducible NOS, conditions that have been suggested to increase sensitivity to NO donors (Nagareddy *et al*., 2005; Williams and Pearce, 2006). The transient increase in oxidative stress and hyperlipidaemia induced by high-fat diet did not cause further reduction in the endothelial function. Remarkably, α-LA was able to reverse the impairment in endothelial vasorelaxation to levels not significantly different from non-diabetic Wistar rats. α-LA

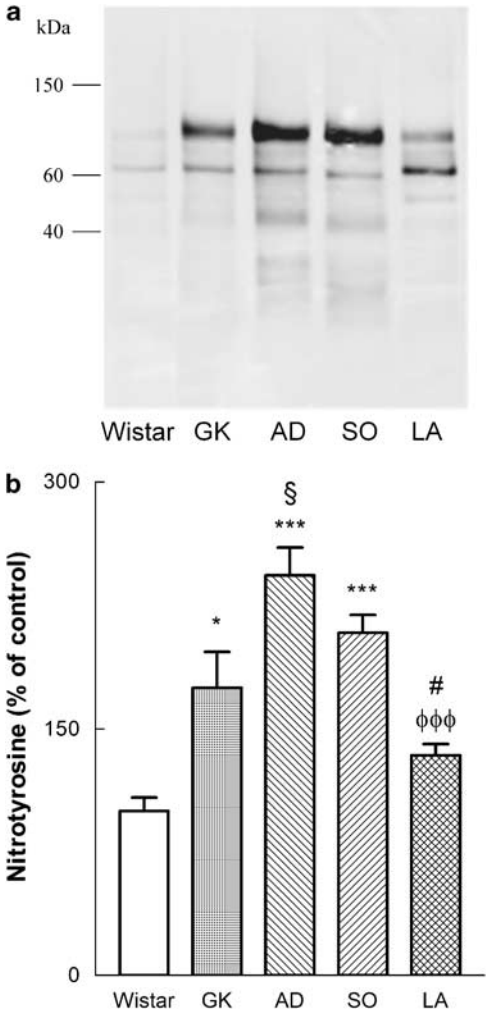

Figure 7 Effects of α-lipoic acid (α-LA) on aortic content of immunoreactive 3-nitrotyrosine during diabetes. (**a**) Representative western blot analyses of 3-nitrotyrosine protein expression in aortas of Wistar and Goto–Kakizaki (GK) rats; 30 μg of protein from aortic lysates was resolved by SDS-polyacrylamide gel electrophoresis (PAGE), transferred to nitrocellulose and probed with 3-nitrotyrosine antibody as described in Materials and methods. (**b**) Averaged densitometric data for diabetic group expressed as a percentage of elevation over the control value established as 100%. Data are presented as mean ± s.e.; of at least seven animals per group. In panel b, $^{*}P<0.05$, $^{***}P<0.001$ vs Wistar group; $^{\S}P<0.05$ vs GK group; $^{\phi\phi\phi}P<0.001$ vs GK + AD group; $^{\#}P<0.05$ vs GK + SO group.

completely decreased all the biomarkers of oxidative stress as well as the hyperlipidaemia and atherosclerotic lesions.

In addition to oxidant-generating pathways characterized in the current work, accumulating studies have suggested that other pathways, such as protein kinase C-dependent signal transduction, aldose reductase and peroxisome proliferator-activated receptor activation (Creager *et al*., 2003; Da Ros *et al*., 2005), may also contribute to development of diabetic vascular diseases. Potential interactions among these pathways are possible and warrant future investigation.

Conclusions

In summary, we have demonstrated that ageing associated with long-term type 2 diabetes induces a progressive depression of endothelial vasodilator responsiveness. This endothelial dysfunction was associated with an apparent decrease in NO production, decrease in the expression of eNOS and with a marked increase in vascular O_2^- production and nitrotyrosine expression. The data from the present study demonstrate a novel role for α-LA in diabetes associated with hyperlipidaemia, with an improvement of endothelial function at least partially attributed to recoupling of eNOS and increased NO bioavailability. The ability of LA to restore endothelial function, decrease systemic and local oxidative stress and lower fasting glycaemia in aged diabetic rats with hyperlipidaemia represents a possible pharmacological approach to treat type 2 diabetes patients and prevent major complications associated with this disease.

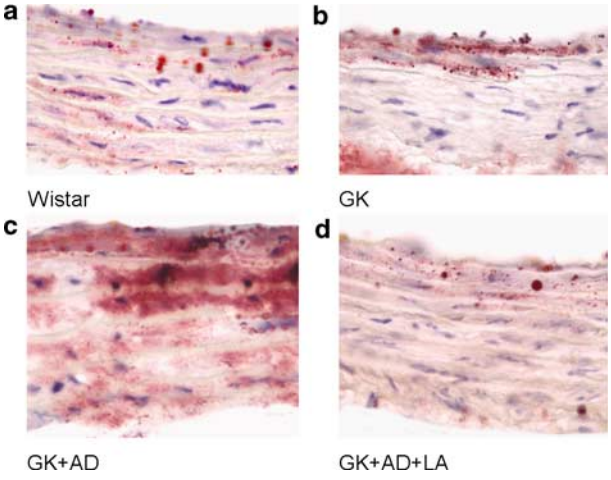

Figure 8 Representative histological staining of aorta from Wistar rats (**a**), Goto–Kakizaki (GK) rats (**b**) and GK rats treated with atherogenic diet (AD) with (**d**) or without (**c**) α-lipoic acid (α-LA). Samples were stained with Oil Red O as described in Materials and methods. GK + AD rats showed marked accumulation, and treatment with α-LA significantly reduced lipid droplets in the vascular wall. The endothelium is facing up in all layers.

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Conflict of interest

The authors state no conflict of interest.

References

Alp NJ, Channon KM (2004). Regulation of endothelial nitric oxide synthase by tetrahydrobiopterin in vascular disease. *Arterioscler Thromb Vasc Biol* **24**: 413–420.

Anter E, Thomas SR, Schulz E, Shapira OM, Vita JA, Keaney Jr JF (2004). Activation of endothelial nitric-oxide synthase by the p38 MAPK in response to black tea polyphenols. *J Biol Chem* **279**: 46637–46643.

Arcaro G, Cretti A, Balzano S, Lechi A, Muggeo M, Bonora E *et al*. (2002). Insulin causes endothelial dysfunction in humans: sites and mechanisms. *Circulation* **105**: 576–582.

Brownlee M (2001). Biochemistry and molecular cell biology of diabetic complications. *Nature* **414**: 813–820.

Burke AP, Kolodgie FD, Zieske A, Fowler DR, Weber DK, Varghese PJ *et al*. (2004). Morphologic findings of coronary atherosclerotic plaques in diabetics: a postmortem study. *Arterioscler Thromb Vasc Biol* **24**: 1266–1271.

Cai H, Harrison DG (2000). Endothelial dysfunction in cardiovascular diseases: the role of oxidant stress. *Circ Res* **87**: 840–844.

Cassini AF, Ferrali M, Pompella A, Maellaro E, Comporti M (1986). Lipid peroxidation and cellular damage in extrahepatic tissue of bromobenzene-intoxicated mice. *Am J Pathol* **123**: 520–531.

Cayatte AJ, Rupin A, Oliver-Krasinski J, Maitland K, Sansilvestri-Morel P, Boussard MF *et al*. (2001). S17834, a new inhibitor of cell adhesion and atherosclerosis that targets NADPH oxidase. *Arterioscler Thromb Vasc Biol* **21**: 1577–1584.

Chang JW, Lee EK, Kim TH, Min WK, Chun S, Lee KU *et al*. (2007). Effects of α-lipoic acid on the plasma levels of asymmetric dimethylarginine in diabetic end-stage renal disease failure on hemodialysis: a pilot study. *Am J Nephrol* **27**: 70–74.

Cheng ZJ, Vaskonen T, Tikkanen I, Nurminen K, Ruskoaho H, Vapaatalo H *et al*. (2001). Endothelial dysfunction and salt-sensitive hypertension in spontaneously diabetic Goto–Kakizaki rats. *Hypertension* **37**: 433–439.

Creager MA, Luscher TF, Cosentino F, Beckman JA (2003). Diabetes and vascular disease: pathophysiology, clinical consequences, and medical therapy. *Circulation* **108**: 1527–1532.

Da Ros R, Assaloni R, Ceriello A (2005). Molecular targets of diabetic vascular complications and potential new drugs. *Curr Drug Targets* **6**: 503–509.

Dalle-Donne I, Rossi R, Giustarini D, Milzani A, Colombo R (2003). Protein carbonyl groups as biomarkers of oxidative stress. *Clin Chim Acta* **329**: 23–38.

de Roos NM, Siebelink E, Bots ML, van Tol A, Schouten EG, Katan MB (2002). Trans monounsaturated fatty acids and saturated fatty acids have similar effects on postprandial flow-mediated vasodilation. *Eur J Clin Nutr* **56**: 674–679.

de Vriese AS, Verbeuren TJ, van de Voorde J, Lameire NH, Vanhoutte PM (2000). Endothelial dysfunction in diabetes. *Br J Pharmacol* **130**: 963–974.

Del Rio D, Stewart AJ, Pellegrini N (2005). A review of recent studies on malondialdehyde as toxic molecule and biological marker of oxidative stress. *Nutr Metab Cardiovasc Dis* **15**: 316–328.

Du XL, Edelstein D, Dimmeler S, Ju Q, Sui C, Brownlee M (2001). Hyperglycemia inhibits endothelial nitric oxide synthase activity by posttranslational modification at the Akt site. *J Clin Invest* **108**: 1341–1348.

Goldberg IJ (2001). Diabetic dyslipidemia: causes and consequences. *J Clin Endocrinol Metab* **86**: 965–971.

Goldberg IJ, Dansky HM (2006). Diabetic vascular disease: an experimental objective. *Arterioscler Thromb Vasc Biol* **26**: 1693–1701.

Goraya TY, Leibson CL, Palumbo PJ, Weston SA, Killian JM, Pfeifer EA *et al*. (2002). Coronary atherosclerosis in diabetes mellitus: a population-based autopsy study. *J Am Coll Cardiol* **40**: 946–953.

Green LC, Wagner DA, Glogowski J, Skipper PL, Wishnok JS, Tannenbaum SR (1982). Analysis of nitrate, nitrite and ^{15}[N] nitrate in biological fluids. *Anal Biochem* **126**: 131–138.

Gryglewski RJ, Palmer RM, Moncada S (1986). Superoxide anion is involved in the breakdown of endothelium derived vascular relaxing factor. *Nature* **320**: 454–456.

Hink U, Li H, Mollnau H, Oelze M, Matheis E, Hartmann M *et al*. (2001). Mechanisms underlying endothelial dysfunction in diabetes mellitus. *Circ Res* **88**: E14–E22.

Kanter JE, Johansson F, LeBoeuf RC, Bornfeldt KE (2007). Do glucose and lipids exert independent effects on atherosclerotic lesion initiation or progression to advanced plaques? *Circ Res* **100**: 769–781.

Kim MS, Park JY, Namkoong C, Jang PG, Ryu JW, Song HS *et al*. (2004). Anti-obesity effects of alpha-lipoic acid mediated by suppression of hypothalamic AMP-activated protein kinase. *Nat Med* **10**: 727–733.

Kobayashi T, Taguchi K, Yasuhiro T, Matsumoto T, Kamata K (2004). Impairment of PI3-K/Akt pathway underlies attenuated endothelial function in aorta of type 2 diabetic mouse model. *Hypertension* **44**: 956–962.

Kocak G, Aktan F, Canbolat O, Ozogul C, Elberg S, Yildizoglu-Ari N *et al*. (2000). alpha-Lipoic acid treatment ameliorates metabolic parameters, blood pressure, vascular reactivity and morphology of vessels already damaged by streptozotocin-diabetes. *Diabetes Nutr Metab* **13**: 308–318.

Lee WJ, Lee IK, Kim HS, Kim YM, Koh EH, Won JC *et al*. (2005). alpha-Lipoic acid prevents endothelial dysfunction in obese rats via activation of AMP-activated protein kinase. *Arterioscler Thromb Vasc Biol* **25**: 2488–2494.

Levine RL, Garland D, Oliver CN, Amici A, Climent I, Lenz AG *et al*. (1990). Determination of carbonyl content in oxidatively modified proteins. *Methods Enzymol* **186**: 464–478.

Lichtenstein AH, Matthan NR, Jalbert SM, Resteghini NA, Schaefer EJ, Ausman LM (2006). Novel soybean oils with different fatty acid profiles alter cardiovascular disease risk factors in moderately hyperlipidemic subjects. *Am J Clin Nutr* **84**: 497–504.

Miller FJ, Gutterman DD, Rios CD, Heistad DD, Davidson BL (1998). Superoxide production in vascular smooth muscle contributes to oxidative stress and impaired relaxation in atherosclerosis. *Circ Res* **82**: 1298–1305.

Molnar J, Yu S, Mzhavia N, Pau C, Chereshnev I, Dansky HM (2005). Diabetes induces endothelial dysfunction but does not increase neointimal formation in high-fat diet fed C57BL/6J mice. *Circ Res* **96**: 1178–1184.

Muller C, Dunschede F, Koch E, Vollmar AM, Kiemer AK (2003). alpha-Lipoic acid preconditioning reduces ischemia-reperfusion injury of the rat liver via the PI3-kinase/Akt pathway. *Am J Physiol* **285**: G769–G778.

Murakami H, Murakami R, Kambe F, Cao X, Takahashi R, Asai T *et al*. (2006). Fenofibrate activates AMPK and increases eNOS phosphorylation in HUVEC. *Biochem Biophys Res Commun* **341**: 973–978.

Nagareddy PR, Xia Z, McNeill J, MacLeod KM (2005). Increased expression of iNOS is associated with endothelial dysfunction and impaired pressor responsiveness in streptozotocin-induced diabetes. *Am J Physiol* **289**: H2144–H2152.

Packer L, Kraemer K, Rimbach G (2001). Molecular aspects of lipoic acid in the prevention of diabetes complications. *Nutrition* **17**: 888–895.

Payne JA, Reckelhoff JF, Khalil RA (2003). Role of oxidative stress in age-related reduction of NO-cGMP-mediated vascular relaxation in SHR. *Am J Physiol* **285**: R542–R551.

Reis ED, Roque M, Dansky H, Fallon JT, Badimon JJ, Cordon-Cardo C *et al*. (2000). Sulindac inhibits neointimal formation after arterial injury in wild-type and apolipoprotein E-deficient mice. *Proc Natl Acad Sci USA* **97**: 12764–12769.

Santos MS, Santos DL, Palmeira CM, Seica R, Moreno AJ, Oliveira CR (2001). Brain and liver mitochondria isolated from diabetic Goto–Kakizaki rats show different susceptibility to induced oxidative stress. *Diabetes Metab Res Rev* **17**: 223–230.

Schalkwijk CG, Stehouwer CD (2005). Vascular complications in diabetes mellitus: the role of endothelial dysfunction. *Clin Sci (Lond)* **109**: 143–159.

Seiça R, Suzuki K, Santos RM, Rosário LM (2004). Deficiência primária da secreção de insulina de ilhéus isolados de ratos Goto–Kakizaki, um modelo animal de diabetes tipo 2 não obesa. *Acta Med Port* **17**: 42–48.

Song KH, Lee WJ, Koh JM, Kim HS, Youn JY, Park HS *et al*. (2005). alpha-Lipoic acid prevents diabetes mellitus in diabetes-prone obese rats. *Biochem Biophys Res Commun* **326**: 197–202.

Vasdev S, Gill V, Parai S, Gadag V (2005). Dietary lipoic acid supplementation attenuates hypertension in Dahl salt sensitive rats. *Mol Cell Biochem* **275**: 135–141.

Visioli F, Smith A, Zhang W, Keaney Jr JF, Hagen T, Frei B (2002). Lipoic acid and vitamin C potentiate nitric oxide synthesis in human aortic endothelial cells independently of cellular glutathione status. *Redox Rep* **7**: 223–227.

Williams JM, Pearce WJ (2006). Age-dependent modulation of endothelium-dependent vasodilation by chronic hypoxia in ovine cranial arteries. *J Appl Physiol* **100**: 225–232.

Yi X, Maeda N (2006). Lipoic acid prevents the increase in atherosclerosis induced by diabetes in apoliprotein E-deficient mice fed high-fat/low-cholesterol diet. *Diabetes* **55**: 2238–2244.

Ziegler D, Gries FA (1997). alpha-Lipoic acid in the treatment of diabetic peripheral and cardiac autonomic neuropathy. *Diabetes* **46**: S62–S66.

Zou MH, Shi C, Cohen RA (2002). Oxidation of the zinc-thiolate complex and uncoupling of endothelial nitric oxide synthase by peroxynitrite. *J Clin Invest* **109**: 817–826.

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