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Lipoic acid effects on established atherosclerosis

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Abstract

Aims—Alpha-lipoic acid (LA) is a commonly used dietary supplement that exerts anti-oxidant and anti-inflammatory effects in vivo and in vitro. We investigated the mechanisms by which LA may confer protection in models of established atherosclerosis.

Main Methods—Watanabe heritable hyperlipidemic (WHHL) rabbits were fed with high cholesterol chow for 6 weeks and then randomized to receive either high cholesterol diet alone or combined with LA (20 mg/kg/day) for 12 weeks. Vascular function was analyzed by myography. The effects of LA on T cell migration to chemokine gradients was assessed by Boyden chamber. NF-κB activation was determined by measuring translocation and electrophoresis migration shift assay (EMSA).

Key findings—LA decreased body weight by $15 \pm 5\%$ without alterations in lipid parameters. Magnetic Resonance Imaging (MRI) analysis demonstrated that LA reduced atherosclerotic plaques in the abdominal aorta, with morphological analysis revealing reduced lipid and inflammatory cell content. Consistent with its effect on atherosclerosis, LA improved vascular reactivity (decreased constriction to angiotensin II and increased relaxation to acetylcholine and insulin), inhibited NF-κB activation, and decreased oxidative stress and expression of key adhesion molecules in the vasculature. LA reduced T cell content in atherosclerotic plaque in conjunction with decreasing ICAM and CD62L (L-Selectin) expression. These effects were confirmed by demonstration of a direct effect of LA in reducing T cell migration in response to CCL5 and SDF-1 and decreasing T cell adhesion to the endothelium by intra-vital microscopy.

Significance—The present findings offer a mechanistic insight into the therapeutic effects of LA on atherosclerosis.

Keywords

Lipoic acid; NF-kappaB; Atherosclerosis; T cells; Innate immunity; Superoxide

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Introduction

α -Lipoic acid (LA; 1,2-dithiolane-3-pentanoic acid) is a naturally occurring disulfide compound commonly found in diet and is a necessary cofactor for mitochondrial enzymes α -ketoglutarate and pyruvate dehydrogenases in mammals (Reed, et al., 1951). Various studies have shown that LA exerts powerful anti-inflammatory and anti-oxidant effects *in vitro* (Packer, et al., 1995). Treatment of cultured monocytes with LA inhibits lipopolysaccharide-mediated induction of pro-inflammatory factors through up-regulation of the PI3 kinase-Akt pathway (Zhang, et al., 2007). Consistent with these effects, LA has been shown to be protective in human diseases associated with abnormal oxidative stress and energy metabolism such as diabetes mellitus (Estrada, et al., 1996; Henriksen, et al., 1997), hyperhomocysteinemia (Baydas, et al., 2002) and hypertension (Vasdev, et al., 2000; Vasdev, et al., 2000). Previous studies performed by our group, have revealed that 4 weeks of dietary supplementation with LA in humans was sufficient to improve endothelial function in patients with metabolic syndrome (Sola, et al., 2005). Prior studies with LA and atherosclerosis have tested the effects of the agent very early on in the life cycle of atherosclerosis development (Zhang, et al., 2008) and have not tested the effects once atherosclerosis is established. In this study, we tested the hypothesis that oral supplementation of LA in the diet would inhibit established atherosclerosis and further investigated the mechanisms by which LA exerts these effects by parallel investigations on vascular inflammation and in particular the infiltration of T cells.

Methods

Organ chamber experiments were performed in the thoracic aorta of WHHL rabbits as detailed in online methods. In-vivo magnetic resonance imaging of the abdominal aorta was performed at various time points as shown in Supplementary Figure 1. Quantitative morphometry, lipid profiles, oxidative stress measures, quantitative real time PCR western, NF- κ B activation assays, Intra-vital microscopy, T-cell migration assays, are all described in the online Supplemental Methods Section.

Statistics

Results are expressed as means $\pm$ SD. The unpaired Student's *t* test was used to compare parameters in the LA and control treated groups. P values < 0.05 were reported as significant. With multiple comparisons, a Bonferroni correction was used for multiple comparisons. In-vitro experiments comparing LA with other anti-oxidants involving multiple groups were analyzed using 1-way ANOVA with a Bonferroni post-hoc correction.

Results

The study design of rabbit experiments is illustrated in Supplementary Figure 1. All rabbits were fed with high cholesterol chow for a period of 6 weeks prior to randomization to LA/control groups in order to hasten the development of plaque in this pre-disposed animal model. During this period the rabbit gained weight (2.62 ± 0.4 kg baseline and 2.96 ± 0.38 kg after 6 weeks of high cholesterol chow; $n = 9$; $P < 0.05$, paired student's *t*-test for 6 weeks versus baseline). Dietary supplementation with LA started at the end of 6 weeks for a duration of 12 weeks, reduced rabbit weight by $15 \pm 4\%$, in contrast control rabbits did not change their weights (Supplementary Figure 2). Compared to the baseline lipoprotein levels, 6 weeks of high cholesterol feeding in the control group significantly increased total, HDL, LDL and VLDL cholesterol. LA did not significantly affect the level of any of the plasma lipoprotein sub-fractions (Supplementary Table 1). LA markedly reduced fasting hyperglycemia and increased insulin level at the end of the treatment period compared to the

control group (Supplementary Table 1). LA treatment did not alter the triglyceride levels after 12 weeks of treatment.

The wall volume in the rabbit abdominal aorta, an indicator of atherosclerotic plaque burden, was analyzed by serial *in vivo* MRI scanning. Comparison with age and sex-matched New Zealand White rabbits confirmed a significantly higher wall volume of abdominal aorta in WHHL rabbits after 6 weeks of feeding with high cholesterol chow (Supplementary Figure 3). After 6 weeks of dietary supplementation with LA, wall volume in the abdominal aorta was significantly lower compared to the control arm (Figure 1A and B). These effects of LA were more pronounced at the end-point (12 weeks). At the end of 12 weeks, there was a significant slowing of the rate of progression of plaque in the LA group, while abdominal aorta wall volume continued to increase in the control group (Figure 1A and B). Morphometric assessment of plaque burden in the thoracic aorta at sacrifice, corroborated the anti-atherosclerotic effects of LA on the abdominal aorta (Figure 1C and D). Immunohistochemical analysis of thoracic aortic sections revealed a reduction in CD68⁺ cells in atherosclerotic plaque and increased smooth muscle content (Figure 2A-D). LA also decreased lipid accumulation and fibrillar collagen deposition (Figure 2E-H).

Figure 3 and Supplementary Table 2 illustrates the responses to various agonists in the thoracic aorta of WHHL rabbits. Figure 3A and B depict LA effects on acetylcholine and insulin mediated responses. In contrast to significant effects on acetylcholine and insulin mediated dilation, LA had no effects in response to SNP, an endothelium-independent vasodilator (Supplementary Table 2). LA reduced vasoconstrictor responses to angiotensin II (Figure 3C) but did not alter responses to phenylephrine or endothelin-1 (Supplementary Table 2). In view of decreased contractility to angiotensin II in the aortic segments, we examined the expression of the angiotensin Type 1 (AT1) receptor. Real time PCR analysis of revealed that AT1 receptor was reduced by LA treatment (Supplementary Figure 4).

LA reduced oxidative stress as evidenced by lower 8-isoprostane levels in plasma and aortic homogenates (Figure 4A and B). These results were confirmed by *in situ* aortic $O_2^{\bullet-}$ measurement using dihydroethidium (DHE) staining (Figure 4C and D). Because various studies have indicated that NADPH oxidase is the major source of vascular $O_2^{\bullet-}$, its activity in aortic homogenates was analyzed. (Mehta and Griendling, 2006) Consistent with the results of 8-isoprostane measurement and DHE staining, basal $O_2^{\bullet-}$ production in aortic homogenates was significantly lower in the LA group compared to the control groups (Figure 4E). In response to exogenously added NADPH, there was a 1.75 fold lower $O_2^{\bullet-}$ generation in LA treated aortas compared to controls (Figure 4E).

As vascular inflammation plays a pivotal role in atherosclerosis we assessed pro-inflammatory gene expression and cellular correlates of inflammation. LA decreased mRNA expression of VCAM-1, ICAM-1 and L-selectin (CD62L) in thoracic aorta (Figure 5A). The mRNA expression of P-selectin and MCP-1 (CCL2) showed non-significant decreases in the LA group (Figure 5A). We corroborated the effect of LA on VCAM-1 and ICAM-1 expression by measuring expression of these adhesion molecules at the protein level (Figure 5B and C). Since NF- κ B regulates expression of pro-inflammatory genes, we examined the content of the p65 sub-unit of NF- κ B in the nuclear and cytosol as a surrogate for its activation. LA decreased the translocation of the p65 sub-unit of NF- κ B from cytosol to nucleus (Figure 5D and 5E). The inhibition of NF- κ B signaling by LA was confirmed by assessing the DNA binding activity of NF- κ B with electrophoretic mobility shift assay (EMSA, Figure 5F and G). Consistent with these effects on NF- κ B, acute treatment with LA inhibited adhesion of leukocytes in the cremasteric circulation when acutely administered to mice 24 hours prior to an inflammatory challenge with TNF α (Supplemental Figure 5).

Since T lymphocytes are critical components of atherosclerotic inflammation, we examined whether supplementation with LA chronically decreased T cell infiltration in aortic wall. Figures 6A and B revealed that LA significantly decreased T cell content (CD3⁺ cells) at the level of the thoracic aorta. Adhesion to endothelium is a very important early step for T cell infiltration. To investigate the effect of LA on the interaction between T cells and endothelial cells, T cells labeled with the fluorescent dye CFSE were injected into high fat fed ApoE^{-/-} mice with or without LA pre-treatment over the preceding 8 weeks. LA pre-treatment significantly decreased the rolling and adhesion of labeled T cells to the cremasteric endothelium (Supplementary Figure 7A and B).

To further investigate the mechanism whereby LA reduces T cell recruitment to atherosclerotic lesions, the effect of LA on transmigration of Jurkat T cells was evaluated using Boyden chamber assays. We assessed the effect of various concentrations of LA on migration of T cells towards a RANTES and SDF-1 gradient. Figures 6C and D demonstrate that LA dose-dependently inhibits transmigration of T cells to RANTES and SDF-1. Finally, we isolated leukocytes from control and LA fed rabbits and examined their migration in response to RANTES and SDF-1 *in vitro*. Fig. 6E and F demonstrated that supplementation with LA decreased the migration of leukocytes in response to RANTES and SDF-1.

DISCUSSION

The present study has multiple findings that may have important implications for the treatment of human atherosclerosis using LA, a commonly used dietary supplement, at low doses. 1) LA decreases atherosclerotic plaque burden within a 12 week period with changes seen as early as 6 weeks; 2) Decreased plaque burden was paralleled by marked improvements in vascular function; 3) LA reduces NADPH oxidase dependent O₂^{•-} and NF-κB-mediated inflammatory responses, offering a molecular basis for the anti-atherosclerotic effects of LA; 4) LA anti-inflammatory effects involves reduction in key adhesion and chemokine molecules involved in T cell trafficking to atherosclerotic plaque; effects that were confirmed by dose dependent effects of LA in reducing T cell migration across CCL5/SDF-1 gradients and prevention of T cells to the endothelium.

MRI scanning demonstrated that 6 weeks of dietary supplementation with LA was sufficient to inhibit atherosclerotic lesion development in WHHL rabbits. We induced rapid progression of plaque by high cholesterol feeding for 6 weeks prior to randomization. LA treatment inhibited progression of plaque in the absence of notable changes in plasma lipoprotein levels. Our data are consistent with the effects noted by Zhang et al but differ in dosing and timing of LA treatment (Zhang et al fed mice with 0.2% (wt/wt) LA for a period of 10 weeks.). In contrast, our studies were in established atherosclerosis with much lower doses (Zhang, et al., 2008). Zhang et al demonstrated an effect of LA in reducing TG concentrations in the ApoE^{-/-} mouse model while total cholesterol increased in the ApoE^{-/-}/LDLR^{-/-} strain. These disparate effects on lipoprotein metabolism raise the question of context dependent effects of LA that may relate to degree and genesis of lipoprotein abnormalities in the animal model studied. In our studies, high fat diet administration in the setting of additional genetic predisposition lead to extremely high values of most lipid parameters and precluded detection of any changes that may have been induced by LA.

An additional important observation in this study pertained to the weight loss noted with LA. LA has been described to have appetite suppressant effect through an AMP kinase mechanism in the hypothalamus (Kim, et al., 2004). The only definitive way to discount an effect on food intake is to perform pair-feeding experiments. Our experiments were very similar to a pair fed experimental situation, in that the LA and control groups consumed equal amount of pre-apportioned diet. Thus the weight loss effects are unlikely to relate to

reduced food intake. While it may be difficult to dissociate the effects on weight loss from its direct effects in reducing plaque progression, we believe that latter effects are distinct and dominant. In the study by Zhang et al that did employ pair feeding, the attenuation of lesion area was not accounted by weight loss alone (Zhang, et al., 2008). Studies by Kim et al in rats, demonstrated weight loss at doses that ranged from 0.25, 0.5 and 1%, wt/wt (Kim, et al., 2004). Our studies were designed cognizant of these earlier studies and therefore employed far lower doses (20 mg/d) than that employed by either study to limit the weight loss effects. Nevertheless it appears that weight loss is seen in the rabbit model and may potentially explain the effects on insulin sensitivity. The effects of LA in potentiating insulin mediated dilation are important as LA has been suggested to be an insulin sensitizing agent through PI3 kinase/Akt dependent pathways (Jacob, et al., 1999; Yaworsky, et al., 2000). LA has been shown to enhance IRS-1 expression and insulin-stimulated IRS-1 association with PI3K (Saengsirisuwan, et al., 2004). Our results indeed do confirm a marked insulin sensitizing effect of LA in the vasculature although. Steady state, levels (non-insulin stimulated) of phosphorylated Akt and eNOS in the aorta were no different. The lack of difference in phosphorylation states by westerns does not by itself argue against the in-vivo functional data as the phosphorylation data was obtained in the absence of agonist stimulation. Thus there could still be important differences in the presence of insulin or acetylcholine. Additionally eNOS and Akt is regulated by phosphorylation at multiple sites and the lack of difference at one site does not preclude differences at other site.

Our results demonstrating a favorable effect on endothelial function is consistent with our prior observations suggesting an effect of LA even in the context of multiple risk factors in diseases such as metabolic syndrome and Type II diabetes (Sola, et al., 2005). Notably, although LA treatment did not affect the vasoconstriction to PE and endothelin-1, it reduced the constriction to angiotensin II through down-regulation of AT1 mRNA, similar to its effects in the renal vasculature (Mervaala, et al., 2003). These effects are consistent with therapeutic synergism seen in our human studies evaluating the effects of AT-1 receptor blockade in conjunction with LA supplementation (Sola, et al., 2005). The renin-angiotensin-aldosterone system, a crucial regulator of vascular homeostasis, has consistently been shown to play a prominent role in atherogenesis (Daugherty, et al., 2000; Munzel, et al., 2008; Weiss, et al., 2001). Conversely, blockade of AT1 receptors in atherosclerosis normalizes NADPH oxidase activity, improves endothelial function, and reduces plaque area and macrophage infiltration (Hornig, et al., 2001; Sorescu, et al., 2001; Warnholtz, et al., 1999). Thus LA may potentially exert additional synergistic effects with drugs that target the RAAS system in atherosclerosis, a finding that may be worthy of exploration in the future.

A novel finding was the pronounced effects of LA on T cell transmigration. Our results show that LA markedly reduced T cell infiltration in the atherosclerotic lesion. This may be attributed to reduced migration or "homing" of T cells presumably through its effects on reducing adhesion molecules such as CD62L (L-Selectin) and ICAM and/or chemotactic influences such as RANTES and SDF-1, factors critically important in the migration of T cell populations to the plaque. It is interesting to note that RANTES is regulated by NFkB and the effects of LA on RANTES expression may relate to these effects (Lee, et al., 2000; Munzel, et al., 2000). These observations are similar to the effect of LA in modulating neural inflammation and reducing the migration of T cells to the central nervous system in experimental encephalomyelitis, through effects on cyclic AMP pathways (Chaudhary, et al., 2006; Schillace, et al., 2007). Thus treatment with LA has broad anti-inflammatory and metabolic effects that may be beneficial in inflammatory diseases.

In conclusion, LA exerts important metabolic and vascular protective effects in models of established atherosclerosis. These findings provide an important impetus to design appropriate human trials with LA.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

LA decreases atherosclerosis burden in WHHL rabbits. A and B, Atherosclerotic burden in abdominal aorta of WHHL rabbits were analyzed by serial MRI at the indicated time points. A representative picture (A) and the summary of the whole group (B) are presented. **, $p < 0.01$ vs control animals. C and D, The descending thoracic aorta of WHHL rabbits were subject to H&E staining, and en-face atherosclerotic area analyzed as described in methods. Two representative pictures (C) and the summary (D) are presented. *, $p < 0.05$ vs control animals.

Figure 2.

LA decreases aortic lipid accumulation and macrophage content. Thoracic aortic sections of WHHL rabbits were stained with anti-CD68 (A, B, and C); anti- α -Actin (D, E, and F); oil red O (G, H, and I); Masson's trichrome (J, K, and L). A representative picture (A, D, G, and J) and the summary (B, E, H, and K as % of wall; C, F, J, and L as % of plaque) are presented. * $p < 0.05$ vs control animals.

Figure 3.

LA improves endothelial function. Thoracic aorta was isolated and mounted on a myograph. Aortic rings were then pre-constricted by PE (0.1 μ M), and the response to graded doses of acetylcholine (A) and insulin (B) were analyzed. Results were expressed as percentage of pre-contraction. $n = 5/\text{group}$. *, $p < 0.05$; **, $p < 0.01$ vs control animals. The constrictive response to angiotensin II (C) and endothelin-1 (D) were analyzed. Results were expressed as percentage of contraction to KCl (120 mM). *, $p < 0.05$ vs control animals.

Figure 4.

LA decreases vascular oxidative stress. A and B, 8-isoprostane levels in plasma (A) and aortic homogenates (B) were measured by EIA. *, $p < 0.05$; **, $p < 0.01$; students' *t* test. C and D, aortic superoxide generation was analyzed by dihydroethidium staining. A representative picture (C) and the summary of results (D) are shown. **, $p < 0.01$; students' *t* test. E, Basal and stimulated superoxide production in aortic tissue lysates was analyzed by Lucigenin chemiluminescence assay. The arrow indicates the addition of NADPH (100 μ M). *, $p < 0.05$; **, $p < 0.01$ vs. control animals.

Figure 5.

LA inhibits inflammatory gene expression and NF- κ B signaling in the vasculature. A, Total RNA were prepared from thoracic aorta of WHHL rabbits. The mRNA expression level of pro-inflammatory genes was analyzed by real-time PCR. *, $p < 0.05$; students't test adjusted by Bonferroni correction. B and C, Total proteins were prepared from thoracic aorta of WHHL rabbits. The protein expression levels of VCAM-1 and ICAM-1 were analyzed by western blot. The western blot (B) and densitometric quantification (C) are shown. *, $p < 0.05$; students't test. D and E, Nuclear and cytosolic proteins were prepared from thoracic aorta of WHHL rabbits. NF- κ B (p65) was analyzed by western blot. The gel image (D) and quantification of western blot results (E) are shown. *, $p < 0.05$; students't test. F and G, Nuclear extracts were prepared from thoracic aorta of WHHL rabbits, the DNA binding activity of NF- κ B was measured with lightshift chemiluminescent EMSA kit. The image (F) and quantification (G) are presented.

Figure 6.

LA decreases T lymphocyte infiltration in atherosclerotic plaque. A and B, immunohistochemical analysis of thoracic aorta with anti-CD3. A representative image (A) and the summary (B) are presented. * $p < 0.05$ vs control animals. C and D, the effect of LA on the transmigration of jurkat T cells in response to chemokine SDF-1 (100ng/ml, C) and RANTES (100ng/ml, D). Data are presented as the mean of 8 wells $\pm$ SD. *, $P < 0.05$; **, $p < 0.01$, student's t test adjusted by bonferroni correction. E and F, the transmigration of leukocytes isolated from control and LA-fed rabbits in response to chemokine SDF-1 (100 ng/ml, E) and RANTES (100 ng/ml, F). *, $P < 0.05$; student's t test.