Lipoic acid, but not tempol, preserves vascular compliance and decreases medial calcification in a model of elastocalcinosis

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Abstract
Vascular calcification decreases compliance and increases morbidity. Mechanisms of this process are unclear. The role of oxidative stress and effects of antioxidants have been poorly explored. We investigated effects of the antioxidants lipoic acid (LA) and tempol in a model of atherosclerosis associated with elastocalcinosis. Male New Zealand white rabbits (2.5-3.0 kg) were fed regular chow (controls) or a 0.5% cholesterol (chol) diet + 10^4 IU/day vitamin D (vitD) for 12 weeks, and assigned to treatment with water (vehicle, n=20), 0.12 mmol·kg⁻¹·day⁻¹ LA (n=11) or 0.1 mmol·kg⁻¹·day⁻¹ tempol (n=15). Chol + vitD-fed rabbits developed atherosclerotic plaques associated with expansive remodeling, elastic fiber disruption, medial calcification, and increased aortic stiffness. Histologically, LA prevented medial calcification by 60% and aortic stiffening by 60%. LA also preserved responsiveness to constrictor agents, while intima-media thickening was increased. In contrast to LA, tempol was associated with increased plaque collagen content, medial calcification and aortic stiffness, and produced differential changes in vasoactive responses in the chol + vitD group. Both LA and tempol prevented superoxide signals with chol + vitD. However, only LA prevented hydrogen peroxide-related signals with chol + vitD, while tempol enhanced them. These data suggest that LA, opposite to tempol, can minimize calcification and compliance loss in elastocalcinosis by inhibition of hydrogen peroxide generation.

Key words: Antioxidants; Vascular calcification; Atherosclerosis; Oxidative stress; Lipoic acid

Introduction
Medial layer vascular calcification and elastocalcinosis decrease arterial compliance and increase morbidity in patients with chronic renal disease, diabetes and other conditions, including aging (1). The mechanisms involved in the pathogenesis of these processes are incompletely understood, but recent research on vascular inflammation has shown that calcification is not simply a passive process, but an active, immune-mediated process (2). Accordingly, another possible factor involved in vascular calcification is oxidative stress. Oxidative stress might result not only from increased inflammation, but also from other factors, including abnormal signaling of calcifying vascular cells (3) and endoplasmic reticulum stress (4).

These characteristics highlight the usual background of atherosclerotic changes in which calcification develops. We recently characterized the role of oxidative stress in the progression of aortic valve calcification (5), while investigating the effects of tempol and lipoic acid (LA). These agents have distinct mechanisms of redox modulation. The former has been described as a superoxide dismutase mimetic that also diverts protein nitration to nitrosation (6). The latter has complex effects that include induction of antioxidant defense enzymes via nuclear factor (erythroid-derived 2)-like 2 (Nrf2) (7). Thus, while tempol appears to directly interact with free radicals, LA appears mainly to interfere with redox signaling pathways.
Experimentally reported effects of tempol include hydrogen peroxide-mediated vasorelaxation (8) and lowering of blood pressure (9). LA can prevent hypertension/hyperglycemia (10) and atherosclerosis (11). Recent data indicate that LA was ineffective in prevention of warfarin-induced medial elastocalcinosis in rats (12), but did prevent vitamin D3-induced calcification via preservation of mitochondrial function and restoration of the Gas6/Axl/Akt survival pathway (13). Both tempol and LA prevent endothelial dysfunction in animal models of vascular disease (14), and LA ameliorates endothelial dysfunction in humans with metabolic syndrome (15). Here, we focused on the vascular effects of LA or tempol in a rabbit model of vascular/aortic valve calcification (16) induced by vitamin D supplementation and a cholesterol-enriched diet. We evaluated the treatment effect on elastocalcinosis associated with atherosclerotic expansive remodeling.

Material and Methods

Reagents

All chemicals, including α-lipoic acid, tempol, cholest erol, superoxide dismutase-polyethylene glycol (PEG-SOD) and PEG-catalase were from Sigma Chemical (USA); 2′,3′-dichlorofluorescein diacetate (DCF) and dihydroethidium (DHE) from Invitrogen (USA); OCT Tissue Tek embebbing compound from Fisher Scientific (USA). RAM11 antibody (dilution 1:200) was from Dako (Denmark). Immunohistochemical reactions were assayed using the Vectastain Elite ABC System (Vector, USA).

Rabbit model

This model was originally described by Drolet et al. (16) as a model of aortic valve calcification. Male New Zealand white rabbits (2.5-3.0 kg) were fed either regular chow (controls, n = 32) or 0.5% cholesterol + 10^4 IU/day vitamin D_3 (choi + vit D) (HCD, n = 20). Additional chol + vitD-fed rabbits were given 0.12 mmol-kg^{-1}day^{-1} lipoic acid (n = 11) or 0.1 mmol-kg^{-1}day^{-1} tempol (n = 15) in drinking water. For comparison purposes, we also studied rabbits fed only a 0.5%-cholesterol diet (n = 9). After 12 weeks, rabbits were anesthetized with 30 mg/kg ketamine and 3.5 mg/kg xylazine, imt. Blood samples for total cholesterol, calcium, phosphorus, glucose, creatinine/urea were collected. Blood pressure was then measured via a catheter that was inserted through the carotid artery and was advanced until reaching the thoracic aorta. After euthanasia with a lethal dose of 100 mg/kg pentobarbital sodium, the descending thoraco-abdominal aorta was collected for further analysis. Most rabbits used in our study were also part of another parallel study focusing on aortic valve calcification (5). This study was approved by an internal scientific institutional committee and by the Ethics Committee of Hospital das Clinicas, Faculdade de Medicina, Universidade de São Paulo (CAPPESQ #254/03), and complied with the Guide for the Care and Use of Laboratory Animals (NIH publication 85-23, rev. 1996).

Vascular ultrasound and radiofrequency measurements

In vivo images of subdiaphragmatic aorta were obtained by a non-invasive high-definition ultrasonography “echotracking” device (Wall-Track System 2, Pie Medical, The Netherlands) as described elsewhere (17). Briefly, two consecutive images of the descending aorta were stored and converted to a radiofrequency signal in a computer. Automatic measurements of internal diameter, wall thickness and beat-to-beat distension (percentage systolic-diastolic variation in internal diameter) were measured. The accuracy of the system was 30 µm for the diastolic diameter measurement and <1 µm for the pulsatile change in diameter. Total vessel diameter (mm) was calculated as the lumen radius + (2 x posterior wall thickness).

Aortic stiffness

Segments of descending thoracic aorta (5 mm) were incubated with 10 µM sodium nitroprusside in 0.9% NaCl, to avoid vascular smooth muscle tone. Then, segments were mounted on an organ-chamber setup and subjected to cumulative distention at a rate of 0.5-mm increments every 30 s, until measurable tension was detected. At this point, the strain (length of distention in mm) was plotted against incremental developed tension (i.e., each 0.5-mm strain vs tension in g). The slope of this line was taken as a measure of aortic stiffness (modified from Ref. 18).

Vessel histology and macrophage detection

Formaldehyde-fixed aortic segments were embedded in paraffin and processed for histology with Von Kossa, Masson’s trichrome, hematoxylin and eosin, and Verhoeff-Van Gieson staining. Macrophages were identified by RAM-11 antibody immunostaining. Collagen deposition, macrophage infiltration and calcification area were calculated in segments of descending thoracic aorta in a blinded manner using the Quantimet analysis software (Leica, Germany).

Organ chamber vascular ring experiments

Vascular reactivity was assessed as described previously (19). Briefly, 5-mm aortic rings were mounted in an organ chamber with Krebs solution (103 mM NaCl, 25 mM NaHCO_3, 11 mM C_6H_12O_6, 4.7 mM KCl, 1.9 mM CaCl_2, 1.2 mM MgSO_4, 1.06 mM KH_2PO_4), pH 7.4, 37°C, aerated with 95% O_2 and 5% CO_2, and attached to a force-displacement transducer (Mp100, Biopac, USA). For isometric tension recording, resting tension was set at 3 g for an equilibration period of 60 min. Precontraction with 0.1 µM noradrenaline was followed by testing.
endothelium-dependent relaxation with acetylcholine (Ach). Contraction in response to 0.12 M KCl and the NO synthase inhibitor Nω-nitro-L-arginine methyl ester (L-NAME, 0.1 mM) was measured to assess maximal vascular contraction and basal NO production, respectively. Endothelium-independent relaxation was assessed with sodium nitroprusside (SNP). The relaxation curves were compared to assess maximum effect and half maximal effective concentration (EC_{50}) dosing.

**In situ aortic ROS microfluorotopography**

*In situ* aortic ROS microfluorotopography was performed with DHE for superoxide and DCF for hydrogen peroxide, as detailed previously (5). Rabbit aorta segments were cryo-cut (30 μm thickness) and incubated with 3 μM DHE or 3 μM DCF; images were obtained with a Zeiss Axiovert 100M scanning confocal microscope and the Axiovision software (Carl Zeiss, Germany). Parallel reading of images was performed with identical laser acquisition settings. The influence of superoxide or hydrogen peroxide on fluorescent signals was assessed via parallel slice incubation with 500 U/mL PEG-SOD or 400 U/mL PEG-catalase.

**Statistical analysis**

Parametric distribution of all variables was assessed with the Shapiro-Wilk test (all samples, <50 per group). Data with a normal distribution are reported as means±SE and were compared with one-way ANOVA, followed by the Student-Newman-Keuls test. For non-parametric distributions of data, variables are reported as box plots with median±interquartile range and range. These data were analyzed with the Kruskal-Wallis test followed by the Dunn test. Significance was considered when P<0.05.

**Results**

Plasma cholesterol, creatinine and calcium-phosphorus product were increased in all chol+vitD-fed rabbits. There were no significant changes in rabbits given tempol or LA. All values were similar to those reported previously (5).

**Lipoic acid prevents increase in vascular stiffness**

While HCD rabbits developed macroscopic enlargement of the arterial tree at several levels, ultrasound/radiofrequency measurements indicated borderline significance for *in vivo* expansive remodeling (Figure 1). Such remodeling was due to increased wall (intimal+medial) thickness with unchanged lumen diameter. There was little change in distension (data not shown). LA-treated rabbits displayed significant expansive vascular remodeling due primarily to vascular wall thickening (Figure 1), contrary to tempol-treated rabbits.

Pulse pressure, a surrogate marker of *in vivo* global arterial stiffness, was augmented in HCD rabbits and the
observed increase was partially inhibited by LA or tempol treatment (Figure 2). However, experiments measuring in vitro aortic stiffness showed a significant increase in HCD rabbit aortas, which was unaltered by tempol treatment. In contrast, HCD lipoic acid rabbit aortas had stiffness levels similar to those from intact control rabbits.

**Lipoic acid, but not tempol, prevented vascular calcification**

Histology confirmed the presence of atherosclerotic plaques in all rabbits given chol + vitD. The plaques were associated with massive calcium deposits and elastic fiber fragmentation/disorganization reminiscent of that observed in human elastocalcinosis in aging, diabetes or renal disease. Effects of tempol or LA in intima and media structures were quite diverse. Treatment with LA did not have any effect on thickness or collagen accumulation in the intima vs HCD rabbits. However, media thickness was significantly increased, in addition to marked prevention of calcification (Figure 3). There was also a non-significant increase (P = 0.10) in plaque macrophage infiltration. In contrast, tempol induced further intimal growth associated with greater collagen accumulation and further enhancement of calcified nuclei in the media, in line with its reported increase in aortic valve calcification (5). Of note, only 27% of aortas from LA-treated rabbits exhibited any measurable (>1% area) medial calcification at histology (P = NS vs controls). In contrast, HCD rabbit aortas showed a 69% incidence of medial calcification vs 73% with tempol. All groups showed similar collagen content in the media. Interestingly, plotting all values of medial calcification in HCD rabbits yield a sigmoidal distribution, reminiscent of the pattern seen in human coronary calcification (20).

For comparison, rabbits given only a cholesterol-enriched diet had increased cholesterol plasma levels and atherosclerotic lesions at histology, but negligible calcification or expansive remodeling, with only minor (7%) wall thickening and unaltered vascular stiffness (data not shown). These findings confirmed that the model described by Drolet et al. (16), but not rabbits fed cholesterol alone, is adequate for vascular calcification and stiffening studies, the focus of the present study.

**Differential effects of lipoic acid and tempol on vascular contractility**

Chol + vitD-fed rabbits had significantly impaired vascular contraction in response to noradrenaline. Remarkably, LA-treated rabbits had preserved contractility, similar to controls. In contrast, tempol significantly decreased the contraction induced by a single concentration of KCl, together with an augmented constriction response to L-NAME, suggestive of excessive basal NO production. While SNP-mediated relaxation was normal in all groups, neither LA nor tempol prevented the impaired Ach-mediated maximal relaxation observed in HCD rabbits. Interestingly, LA-treated rabbits had a lower sensitivity to Ach-mediated relaxation than the other

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**Figure 2.** A, B and C, Systolic blood pressure, diastolic pressure and pulse pressure measured with direct catheterization in anesthetized rabbits immediately before euthanasia. Heart rate was not different among groups. C: controls (n = 28); HCD: 0.5% cholesterol + vitamin D feeding (n = 16); HCDT: HCD + tempol (n = 10); HCDLA: HCD + lipoic acid (n = 7). D, *In vitro* arterial stiffness assessed as the slope of the strain-tension relationship in rabbit aortic segments. C (n = 20); HCD (n = 13); HCDT (n = 13); HCDLA (n = 10). See Material and Methods for details. Data are reported as means ± SE. *P < 0.05 vs C; †P < 0.05 vs HCDLA (Student-Newman-Keuls test).
chol+vitD-fed rabbits, without or with tempol supplementation. This was evident from higher EC50 values (median EC50 – 6.47 vs – 7.28 or – 7.24 M, respectively, P, 0.05).

This is consistent with preservation of contractile capacity in LA-treated rabbits, thus requiring increased Ach concentrations for similar degrees of relaxation (Figure 4).

**Lipoic acid or tempol differentially modulated redox processes**

In HCD rabbits, DHE signals exhibited a patchy aspect with strong concentration mainly around medial calcification nuclei. Tempol robustly reduced DHE fluorescence to levels below even controls, but significantly increased DCF-derived signals, indicating increased H2O2 production, a result in line with previous data (5,8). LA also partially decreased DHE-derived signals but, contrary to tempol, promoted a consistent decrease in DCF fluorescence, which was similar to controls (Figure 5). Control experiments with PEG-SOD and PEG-catalase revealed robust decreases in fluorescence signals with DHE and DCF, respectively (data not shown).

**Discussion**

In the present study, we showed that LA protected against medial vascular calcification and prevented the associated loss of arterial compliance and contractility, despite increasing arterial wall thickness. This increased artery wall thickness observed by ultrasound in LA-treated rabbits was due mainly to increased medial, rather than intimal, thickness as was demonstrated by histology. In contrast, tempol did not have such a preventive effect, and actually potentiated the increase in vascular calcification.

The HCD rabbit model, in addition to displaying aortic valve calcification (16), also develops complex vascular disease including atherosclerosis with lumen diameter preservation (expansive remodeling), medial calcification and arterial stiffening. The vascular hypocontractility seen in the model could be explained either by a direct vitD effect (21) or medial calcification, with structural derangement of the medial layer. Such alterations, at least in part analogous to those described in human elastocalcinosis, differ substantially from those found in hypercholesterolemic rabbits, which do not display extensive calcification of the medial layer or vascular remodeling. These structural changes can also explain the differences in contraction responses to NA or KCl in this model, and those reported in the literature on hypercholesterolemic rabbits.

Multiple, opposite effects of different antioxidants should primarily reflect their different mechanisms of action. Tempol is a nitroxide that targets ROS and reactive nitrogen species indirectly (6), although its mechanism of action is debatable. The usually described SOD-mimetic activity may not be its major mechanism of action.
action, which instead may involve a rapid reaction of tempol with nitrogen dioxide or carbonate radical (22). We recently reported that aortic segments from HCD rabbits showed increased Nox4, but not Nox1 NADPH oxidase isofrom mRNA expression, and that tempol, but not LA, increased tempol (n = 22); HCD: 0.5% cholesterol + vitamin D feeding (n = 13); HCDT: HCD + tempol (n = 12); HCDLA: HCD + lipoic acid (n = 10). See Material and Methods for details. In A and B, data are reported as means ± SE and parametric tests one-way ANOVA followed by the Student-Newman-Keuls test were used. In C, D, and E, data are reported as median ± interquartile ranges, with range also reported in C, and non-parametric tests Kruskal-Wallis followed by the Dunn test were used. *P<0.05 vs C; **P<0.05 vs HCD; †P<0.05 vs HCDT; **P<0.05 for HCDLA vs HCD or HCDT for EC50 of Ach-induced relaxation.

In our model, the final effect of LA was to decrease superoxide, and particularly hydrogen peroxide, signals. This further highlights a role for hydrogen peroxide in vascular dysfunction and calcification, which could reflect some known opposite effects of either species on vascular smooth muscle cell (VSMC) signaling, with superoxide associated mainly with proliferation and hydrogen peroxide with apoptosis (25). Moreover, recent observations showed that hydrogen peroxide plays a key role in VSMC osteogenic differentiation, via Akt-mediated induction of Runx2 transcription factor (26). Our previous results in this model showed that tempol, but not LA, increased Nox4 expression (5). This NADPH oxidase isofrom appears to preferentially generate hydrogen peroxide rather than superoxide (23) and is associated with decreased VSMC proliferation and differentiation (27). A peculiar aspect of our DCF signals (Figure 5) was their close association with elastic fibers. Interestingly, oxidative stress promotes (28) elastic network disorganization and elastocalcinosis. A role for oxidative stress in the calcification model is further corroborated by the effects of hydrogen peroxide and catalase addition in the in vitro calcification model (5).
Our data are in agreement with and extend the report of Kim et al. (13), who recently demonstrated that LA inhibited in vitro and vitamin D3-induced in vivo vascular calcification by recovering mitochondrial function and restoring the Gas6/Axl/Akt survival pathway. On the other hand, our results are at variance with findings by Lalaoui et al. (12) and Yamada et al. (29). Lalaoui observed no effect of LA in a rat model of warfarin-induced medial elastocalcinosis, and Yamada et al. reported that tempol was able to reduce arterial calcification by ~33% in uremic rats. Differences in species, doses and time of antioxidant administration are possible reasons for these contrasting data. Furthermore, specific aspects of each calcification model should be considered. Warfarin inhibits carboxylation-driven activation of the Matrix Gla protein, a natural inhibitor of vascular calcification, and thus exploits a specific pathway of the calcification process per se. In comparison, our vitD model explores several mechanisms present in complex human vascular disease that drive medial calcification, in particular, inflammation and oxidative stress. In the warfarin model used by Lalaoui, there was a small elevation of aortic superoxide, while hydrogen peroxide levels were not assessed. In the present study, there was a marked increase in oxidant generation, and while tempol inhibited superoxide generation, it could not prevent elastocalcinosis. These opposing effects of tempol suggest that distinct redox signaling pathways have divergent effects on vascular calcification. Comparable considerations can be made for the research by Yamada et al. (29). Overall, combining the production and elimination of diverse intermediates such as superoxide and hydrogen peroxide under the label of “ROS” or “oxidative stress” is actually not accurate. They have distinct physicochemical properties and reactivities, and can have opposite effects on basic cellular functions, e.g., apoptosis (25). Grouping compounds with distinct effects, such as tempol and LA, together under the label of “antioxidants” also seems an oversimplification. Specific effects of some compounds, such as the increase in hydrogen peroxide with tempol, may contribute in some cases to collateral signaling, which in our study translated into enhanced calcification.

Clinical studies with antioxidants have resulted in considerable controversy, with somewhat conflicting but largely negative results (30). In this context, our study has potential implications. First, the effect of LA indicates that redox processes have a mechanistic role in vascular calcification and that appropriate interventions to correct abnormal signaling can result in a novel perspective to control or limit medial calcinoses in a variety of clinical settings. A parallel implication is that, despite being grouped together as antioxidants, tempol and LA acted in distinct pathophysiological directions, and support an overall conclusion that the effect of a given “antioxidant” will be highly specific for each compound and situation, thus negating a putative “class effect” based solely on antioxidant properties.

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Figure 5. Upper panels, DHE fluorescence (red) in control and HCD-fed rabbits in the absence or presence of tempol or lipoic acid (LA). Please note increased signals around calcification nuclei (asterisks). Lower panels, DCF fluorescence (green) in control and 0.5% cholesterol+vitamin-fed rabbits in the absence or presence of tempol or LA. Controls with polyethylene glycol (PEG)-superoxide dismutase or PEG-catalase showed respective decreases in DHE or DCF signals (data not shown). C: controls; HCD: 0.5% cholesterol+vitamin D feeding; HCDT: HCD feeding+tempol; HCDLA: HCD feeding+LA; DCF: 2',3'-dichlorofluorescein diacetate; DHE: dihydroethidium. Data are from 3 or more rabbits for each group. Magnification bar = 50 μm.
References


