Protective effect of α -lipoic acid against antimycin A cytotoxicity in MC3T3-E1 osteoblastic cells

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Abstract Oxidative stress represents a major cause of cellular damage and death in the process of osteoporosis. Antimycin A (AMA) has been shown to stimulate mitochondrial superoxide anions and reactive oxygen species (ROS). α -Lipoic acid (α -LA) is a naturally occurring essential coenzyme in mitochondrial multienzyme complexes and acts as a key player in mitochondrial energy production. However, whether α -LA affects the cytotoxicity of AMA in osteoblastic cells is unknown. In this study, we investigated the protective effects of α -LA against AMA-induced cytotoxicity using the MC3T3-E1 osteoblast-like cell line. Our results indicated that α -LA treatment attenuated AMA-induced cytotoxicity and LDH release in a dose-dependent manner. Notably, a significant recovery effect of α -LA on mineralization inhibited by AMA was found. Our results also demonstrated that treatment with 50 μ M AMA leads to a reduction of mitochondrial membrane potential (MMP) and the complex IV dysfunction, which was inhibited by pretreatment with α -LA in a dose-dependent manner. In addition, treatment with α -LA significantly reduced the generation of ROS and mitochondrial superoxide production induced by AMA. In addition, our result suggests that PI3K/Akt and CREB pathways are related to the protective effect of α -LA. Importantly, Hoechst 33258 staining results indicated that pretreatment with α -LA prevented AMA-induced apoptosis. Mechanistically, we found that α -LA prevents MC3T3-E1 cells from apoptosis through

attenuating cytochrome C release and reducing the level of cleaved caspase-3.

Keywords Antimycin A · α -Lipoic acid · Mineralization · Oxidative stress · Mitochondrial dysfunction

Introduction

Osteoporosis, one of the most common aging-related bone diseases, is a systemic skeletal disease characterized by low bone mass and microarchitectural deterioration of bone tissue, with a consequent increase in bone fragility (Ahmed and Elmantaser 2009). The cause of osteoporosis remains elusive. Oxidative stress represents a major cause of cellular damage and death in a plethora of pathological conditions, including osteoporosis. Markedly elevated levels of oxidative stress markers have been found in the blood (Forrest et al. 2005). In vitro studies have shown that oxidative stress inhibits osteoblastic differentiation (Bai et al. 2004) and induces osteoblast insults and apoptosis (Fatokun et al. 2006). Notably, increasing evidence has shown that mitochondrial energy metabolism is extremely sensitive to impairment by free radicals, and mitochondrial oxidative stress limits metabolic recovery (Fiskum et al. 2004). Inhibitors of the mitochondrial electron transport chain have been reported to induce cell injuries in vitro and in vivo (Brouillet et al. 1993; Smith and Bennett 1997). Antimycin A (AMA) has been shown to be an inhibitor of mitochondrial complex III in the electron transport chain. Treatment with AMA has been shown to stimulate mitochondrial superoxide anions and consequential H_2O_2 production (Aon et al. 2003), affording a means of augmenting mitochondrial reactive oxygen species (ROS).

α -Lipoic acid (α -LA), also called thioctic acid, is chemically named 1,2-dithiolane-3-pentanoic acid ($C_8H_{14}O_2S_2$),

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which can be detected naturally in fruits, vegetables, and synthesized in animals and humans. α -LA is a naturally occurring essential coenzyme in mitochondrial multienzyme complexes and acts as a key player in mitochondrial energy production (Nagesh Babu et al. 2011). Multiple lines of evidence have demonstrated its multifunctional antioxidant activities in the prevention or treatment of pathological conditions mediated via oxidative stress (Packer et al. 1995; Biliska and Wlodek 2005). It has also been shown that α -LA can effectively inhibit ROS-related pathologies, such as ischemia–reperfusion injury (Coombes et al. 2000) and ovariectomy-induced osteoporosis (Polat et al. 2013). A model commonly used to study osteogenic development is the MC3T3-E1 osteoblast-like cell line. The MC3T3-E1 cell culture system represents a very useful model for studying the process of osteoblast function (Quarles et al. 1992). Therefore, we investigated the protective effects of α -LA against AMA-induced cytotoxicity using osteoblast-like MC3T3-E1 cells.

Materials and methods

Cell culture and treatment

Murine osteoblastic MC3T3-E1 cells were cultured at 37 °C in 5 % CO₂ atmosphere in α -Minimum Essential Medium (MEM) containing 10 % heat-inactivated fetal bovine serum (FBS), 100 U/ml penicillin, and 100 μ g/ml streptomycin. When cells were grown to confluence, 10 mM β -glycerophosphate and 50 μ g/ml ascorbic acid were added to medium to initiate differentiation. After 3 days, the cells were preincubated with α -LA for 12 h at the concentrations of 10, 50, and 100 μ M before treatment with 50 μ M AMA for 24 h.

Measurement of ATP level and mitochondrial complex IV activity

The intracellular ATP level was determined by luciferase reaction using a commercial ATP Assay Kit (BioAssay Systems, USA). Cytochrome C oxidase activity was determined by the commercial microplate assay (MitoSciences, USA). Protein concentrations determined by using the Bio-Rad protein assay reagent were used for internal control.

Cell viability measurement

Cell viability was determined via reduction of 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) by NAD-dependent dehydrogenase activity to form a colored reaction product. Upon completion of treatment, MTT (0.5 mg/ml in PBS) was added to each well, and the plates were incubated for an additional 2 h. After the removal of solutions in the well, dimethyl sulfoxide was added to dissolve

formazan products. Absorbance of each well recorded at 570 nm on a microplate spectrophotometer was used to index cell viability.

Lactate dehydrogenase release determination

Extracellular lactate dehydrogenase (LDH) that is released into the culture medium is a marker for cell damage. Upon completion of treatment, LDH activity measurements were performed using a commercially available assay (Roche Applied Science). Absorbance recorded at 490 nm was used to index amount of LDH release. The ratio of LDH activity in the supernatant to the total LDH activity was calculated to reflect the percentage of cell death.

Mitochondrial membrane potential determination

The fluorescence dye tetramethylrhodamine methyl ester (TMRM) was used to measure mitochondrial membrane potential (MMP) according to the manual instructions. Upon completion of treatment, cells were loaded with 20 nmol/L TMRM (Invitrogen, USA) for 60 min at RT. Fluorescence signals were captured using a fluorescence microscope, and the average fluorescence intensity analyzed by the Image-Pro Plus software was used to calculate the level of MMP.

Determination of mitochondrial ROS

Mitochondrial ROS was measured using MitoSOX Red (Invitrogen, USA) according to the manufacturer's instructions. Upon completion of the indicated treatment, cells were loaded with 5 μ M MitoSOX Red in Hank's balanced salt solution (HBSS) and incubated for 10 min at 37 °C. Fluorescence signals were recorded by a fluorescence microscope.

Phosphatidylinositol-3-kinase activity

Phosphatidylinositol-3-kinase (PI3K) activity in cell lysates was determined using a PI3 kinase ELISA kit (Echelon Biosciences, USA) according to the manufacturer's instructions. The colorimetric signal was used to index PI3K activity.

Morphological analysis of apoptosis

The patterns of apoptosis were determined by Hoechst 33258. Briefly, cells were loaded with 50 μ g/ml Hoechst 33258 and incubated at 37 °C for 30 min in darkness. Cells were washed for three times. Chromatin condensation was observed by confocal microscopy. Apoptotic cells showed blue nuclear condensation and fragmentation. The fractions of apoptotic cells were determined relative to total cells. At least 200 cells were counted in each experiment.

Western blot analysis

Cells were lysed in cell lysis buffer (Cell Signaling, USA) containing protease inhibitor cocktail (Calbiochem, USA). Same amount of protein extracts (20 μ g) were separated by SDS-PAGE (12 % Bis-Tris gel, Invitrogen, USA) and transferred to hydrophobic polyvinylidene fluoride (PVDF) membranes. Membranes were blocked with 5 % nonfat dry milk in TBST buffer (20 mM Tris-HCl, 150 mM sodium chloride, 0.1 % Tween-20) followed by being sequentially incubated with primary antibodies and with corresponding HRP-linked secondary antibodies. Blots were developed using Western Lightning Plus-ECL, Enhanced Chemiluminescence Substrate (Thermo Fisher Scientific, MA, USA). The images were captured and analyzed using Amersham HyperfilmTM ECL (GE Healthcare, München, Germany). The analysis of band intensities was performed using the software ImageJ (NIH, USA). The primary antibodies used in this study are as follows: The antibodies against cytochrome C oxidase subunit IV (COX4) (no. sc-133478), and β -actin (no. sc-130656) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The primary antibodies against cleaved caspase-3 (no. 9579), p-creb (no. 9198), p-Akt (no. 4060), and total Akt (no. 4691) were obtained from Cell Signaling Technology, USA. The secondary antibodies used in this study are as follows: The HRP-linked anti-rabbit IgG antibody (no. 7074) and the HRP-linked anti-mouse IgG antibody (no. 7076) were obtained from Cell Signaling Technology, USA.

Reactive oxygen species determination

Intracellular ROS was measured by using a nonpolar compound that is converted by cellular esterases to polar and membrane impermeable derivative fluorescence dye 2',7'-dichlorofluorescein diacetate (DCFH-DA). Upon completion of drug treatment, cells were loaded with 10 μ M DCFH-DA (Sigma, USA) and incubated in a CO₂ incubator for 1 h at 37 °C. In the presence of intracellular ROS, DCFH is oxidized to highly fluorescent 2',7'-dichlorofluorescein (DCF). Fluorescence signals were captured using a fluorescence microscope, and the level of average fluorescence intensity analyzed by Image-Pro Plus software was used to present intracellular ROS.

Measurement of cytochrome C release

The release of cytochrome C from mitochondria to cytosol was determined by a cytochrome C detection assay. Briefly, cells were treated with an ice-cold cytosolic extraction buffer (250 mM sucrose, 20 mM Hepes pH 7.4, 10 mM KCl, 1 mM EGTA, 1 mM EDTA, 1 mM MgCl₂, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 1 mM benzamidine, 1 mM pepstatin A, 10 mg/ml leupeptin, and 2 mg/ml

aprotinin) on ice for 30 min followed by gentle homogenization using a glass Dounce homogenizer. After centrifugation at 2500 rpm at 4 °C for 15 min, lysate supernatants were collected and centrifuged at 15,000 rpm at 4 °C for 30 min in order to yield a cytosolic extract. The cytosolic extract was collected and used for measuring cytochrome C levels by western blot analysis.

Statistical analysis

Results obtained from different experiments were presented as mean $\pm$ standard error of mean (SEM) of separate experiments ($n \geq 3$). Data were analyzed using analysis of variance (ANOVA), followed by Tukey–Kramer multiple comparison (Prism 5, GraphPad Software, La Jolla, CA, USA). Difference between two treatments was considered to be statistically significant at $P < 0.05$.

Results

Effects of α -LA on AMA-induced cell death

The concentration of α -LA we used in this study was screened before AMA treatment experiment. To study the effect of α -LA on osteoblastic MC3T3-E1 cells, cells were treated with increasing concentrations of α -LA (0 nM, 10 nM, 100 nM, 1 μ M, 10 μ M, 50 μ M, 100 μ M, 1 mM, or 5 mM) in the absence of AMA treatment. Cell viability, intracellular ROS, and MMP of osteoblastic MC3T3-E1 cells were not significantly changed after treatment with α -LA under 1 mM (data not shown). Therefore, in this study, cells were treated with α -LA at the concentrations of 10, 50, and 100 μ M.

Firstly, we determined whether α -LA inhibits cell death induced by AMA, a mitochondrial inhibitor that increases the generation of ROS. When the cells were pretreated with α -LA at the concentrations of 10, 50, and 100 μ M in the presence of 50 μ M AMA, α -LA significantly suppressed the AMA-induced cytotoxicity in a dose-dependent manner (Fig. 1a). LDH is a soluble enzyme located in the cytosol. The enzyme is released into the surrounding culture medium upon cell damage. LDH activity in the culture medium can be used as a biomarker of a measurement of cytotoxicity. To confirm that α -LA mitigated cell vulnerability to AMA, the levels of cellular toxicity were determined using an LDH assay. As expected, α -LA treatment in MC3T3-E1 cells significantly reduced AMA-induced LDH release in a dose-dependent manner (Fig. 1b). These results suggest that α -LA could protect MC3T3-E1 cells against AMA-induced cell death.

Fig. 1 Effects of α -LA on AMA-induced impairment in cell viability and LDH release. Osteoblasts (5×10^6) were preincubated with α -LA for 12 h before treatment with 50 μ M antimycin A (AMA) for 24 h. **a** Cell viability was determined by MTT assay; OD value at 570 nm was

determined by a microplate spectrophotometer. **b** Patterns of LDH release (* $P < 0.01$ vs NS group; # $P < 0.01$ vs AMA-treated group; experiments were repeated for four times)

Effect of α -LA on the osteoblast dysfunction induced by AMA

To investigate the effect of α -LA on the osteoblast dysfunction induced by AMA, we measured calcium deposition using Alizarin Red staining. Our results indicated a significant recovery effect of α -LA in a dose-dependent manner on mineralization inhibited by AMA (Fig. 2). This result suggests that α -LA attenuates AMA-induced dysfunction of osteoblasts.

Effect of α -LA on AMA-induced mitochondrial dysfunction in osteoblastic MC3T3-E1 cells

To investigate the effects of α -LA on mitochondrial function of osteoblasts, we measured MMP and complex IV activity after osteoblasts were treated with AMA in the presence or absence of α -LA. TMRM is a fluorescent probe that is accumulated in mitochondria depending on the MMP. In this study, MMP was measured by using TMRM staining after osteoblasts were

Fig. 2 Effect of α -LA on the osteoblast dysfunction induced by AMA. Osteoblasts (5×10^6) were preincubated with α -LA for 12 h before treatment with 50 μ M antimycin A (AMA) for 24 h. Data are expressed as a percentage of control (* $P < 0.01$ vs NS group; # $P < 0.01$ vs AMA-treated group; experiments were repeated for five times)

stimulated with AMA following pretreatment with α -LA (Fig. 3a). Our results demonstrated that AMA leads to a decrease in MMP, which was inhibited by treatment with α -LA (10, 50, 100 μ M). We then examined the activity of mitochondrial respiratory chain complex IV. AMA led to the reduction of this activity; however, α -LA (10, 50, 100 μ M) substantially sustained the activity even in the presence of AMA (Fig. 3b). These findings suggested that the cytoprotective effect of α -LA resulted from mitochondrial protection.

α -LA reduces the production of reactive oxygen species induced by AMA

Increased mitochondrial free-radical production has traditionally been attributed to deficits in the electron transport chain function. To investigate whether the cytoprotective action of α -LA is related to its antioxidant activity, we assessed the changes of ROS release using the fluorescent probe DCFH-DA. Our results indicated that treatment with α -LA significantly reduced the generation of ROS induced by AMA (50 μ M) in a dose-dependent manner. To further assess the effect of α -LA (10, 50, 100 μ M) on mitochondrial superoxide production, we measured mitochondrial superoxide using MitoSOX, a live-cell-permeable and mitochondrial localizing superoxide indicator. As shown in Fig. 4b, AMA treatment significantly increased mitochondrial MitoSOX fluorescence; however, pretreatment with α -LA (10, 50, 100 μ M) substantially inhibited the stimulatory effect of AMA to enhance mitochondrial superoxide production.

Effect of α -LA on PI3K activity and CREB phosphorylation of osteoblasts in the presence of AMA

Antimycin A induced inactivation of PI3K and cAMP response element-binding protein (CREB), which are known to function as pro-survival signals. Therefore, we determined whether PI3K and CREB activation are related to the cytoprotective effect of α -LA in osteoblastic MC3T3-E1

Fig. 3 Effect of α -LA on AMA-induced mitochondrial dysfunction in osteoblastic MC3T3-E1 cells. **a** Osteoblasts (1×10^6) were preincubated with α -LA for 12 h before treatment with 50 μ M antimycin A (AMA) for 24 h. Mitochondrial membrane potential (MMP) was determined by tetramethylrhodamine methyl ester (TMRM). **b** Osteoblasts (5×10^6) were preincubated with α -LA for 12 h before treatment with 50 μ M antimycin A (AMA) for 24 h. Complex IV activity was determined by the commercial microplate assay (* $P < 0.01$ vs NS group; # $P < 0.01$ vs AMA-treated group; experiments were repeated for five times)

cells. PI3K activity was measured after treatment with AMA (50 μ M) in the absence or presence of α -LA. It was shown that AMA decreased PI3K activity and pretreatment with α -LA (50 μ M) prevented the decline of PI3K activity in cells (Fig. 5a). CREB is an important phosphorylated target of PI3K. To further demonstrate that the CREB is biologically relevant, we investigated the effects of α -LA on the phosphorylation of CREB. Treatment with AMA (50 μ M) decreased phosphorylation of CREB, and preincubation with α -LA (50 or 100 μ M) enhanced the phosphorylation of CREB in the presence of AMA (Fig. 5b). This result suggests that PI3K and CREB are related to the protective effect of α -LA. The protein kinase B (Akt) could be phosphorylated and activated by extracellular factors in a PI3K-dependent fashion. Akt participates in a variety of important physiological functions through phosphorylation. Specifically, the activated PI3K/Akt pathway was involved in regulating cell survival. Our results indicated that AMA significantly reduced the levels

Fig. 4 α -LA reduces the production of reactive oxygen species (ROS) induced by AMA. Osteoblasts (1×10^6) were preincubated with α -LA for 12 h before treatment with 50 μ M antimycin A (AMA) for 24 h. **a** The data shows changes in levels of ROS, which was measured by DCFH fluorescence method. **b** Mitochondrial superoxide levels were detected using MitoSOXTM Red mitochondrial superoxide indicator (* $P < 0.01$ vs NS group; # $P < 0.01$ vs AMA-treated group; experiments were repeated for four times)

of phosphorylated Akt, which was rescued by α -LA in a dose-dependent manner (Fig. 5c).

Effect of α -LA on AMA-induced apoptosis in osteoblastic MC3T3-E1 cells

To investigate the effects of α -LA on apoptosis in osteoblasts, patterns of apoptosis were determined by Hoechst 33258 staining. Results indicated that the nuclear condensation characteristic of apoptosis was increased in MC3T3-E1 cells after AMA treatment. However, α -LA significantly reduced the number of apoptotic cells in response to AMA treatment (Fig. 6).

Fig. 5 Effect of α -LA on PI3K activity and CREB phosphorylation of osteoblasts in the presence of AMA. Osteoblasts (5×10^6) were preincubated with α -LA for 12 h before treatment with 50 μ M antimycin A (AMA) for 24 h. **a** PI3K activity in cell lysates was evaluated using ELISA assay. **b** Phosphorylated CREB was evaluated using cell-based phospho-CREB (S133) western blot analysis. **c** Phosphorylated Akt was evaluated using western blot analysis (* $P < 0.01$ vs NS group; # $P < 0.01$ vs AMA-treated group; experiments were repeated for three to five times)

Dysfunction of mitochondria and impaired MMP led to the release of cytochrome C into the cytoplasm and the activation of caspase-3 (Saelens et al. 2004). Our results indicated that cytochrome C in cytosol is obviously augmented in MC3T3-E1 osteoblastic cells after AMA treatment, which is significantly prohibited by α -LA treatment (100 μ M). Mitochondrial protein COX4 was used as the negative control for cytosolic fractions (Fig. 7a). It was also shown that cleaved caspase-3 is increased in MC3T3-E1 osteoblastic cells after AMA treatment and that the activation of caspase-3 is obviously prevented by α -LA treatment (Fig. 7b).

Discussion

We demonstrated in the present study that α -LA pretreatment resulted in a significant attenuation of AMA-induced

cytotoxicity in MC3T3-E1 osteoblastic cells. In osteoblasts, previous studies have shown that AMA induced cell apoptosis via a mitochondria-dependent pathway. Notably, AMA can decrease osteoblast function by inhibiting osteoblast growth and mineralization (Choi and Lee 2011). In this study, α -LA showed a recovery effect on mitochondrial defects induced by AMA. Osteoblast mitochondria play a vital role in calcium transport and the calcification of the extracellular matrix (Stambough et al. 1984). Mineral formation occurs in matrix vesicles and within mitochondria. Release of mitochondrial calcium and mineral ion loading of matrix vesicles plays a vital role in mineralization. Moreover, mitochondrial respiratory chain complexes regulate mineralization through controlling the levels of the matrix vesicle content of inorganic pyrophosphate (PPi) and the calcium-precipitating ability associated with matrix vesicles (Wuthier et al. 1985). Mitochondrial dysfunction leads to rapid release of mitochondrial Ca^{2+} into

Fig. 6 α -LA ameliorated AMA-induced apoptosis in osteoblastic MC3T3-E1 cells. **a** MC3T3-E1 cells (1×10^6) were pretreated with α -LA for 12 h before treatment with 50 μ M antimycin A (AMA) for 24 h, and then cells were stained with Hoechst 33258 to assess morphological valuation of apoptosis. Apoptotic cells were recognized by condensed/fragmented nuclei. **b** Statistical analysis of apoptotic cells (* $P < 0.01$ vs. control group; # $P < 0.01$ vs. AMA-treated group; experiments were repeated for four times)

the cytosol, which may cause local precipitation of calcium phosphate and thus be coupled with formation of matrix vesicles (Boonrungsiman et al. 2012). In this study, our results indicate that AMA impairs MMP and complex IV activity. These results suggest that AMA might inhibit mineralization by causing mitochondrial dysfunction. Therefore, the recovery of AMA-induced mitochondrial dysfunction by α -LA may have an influence on the osteoblast dysfunction induced by AMA.

Previous studies have demonstrated that aging, estrogen deficiency, and inflammatory diseases are the most common factors contributing to the development of osteoporosis (Mundy 2007). Alterations in the electron transport chain by AMA lead to over-generation of superoxide radical anions. Mitochondrial oxidative stress has been attributed to respiratory complex anomalies, genetic defects, or insufficient oxygen or glucose supplies. Superoxide is the proximal oxidant that leads to the formation of other deleterious oxidants. This molecule can cause additional damage within the cell by entering the intermembrane space through the mitochondrial transition pore and/or through voltage-gated anion channels (Turrens 2003). Notably, mitochondrial ROS production in response to the aging process has been associated with the progression of osteoporosis (Potenza et al. 2009). In this study, the potential protective effects of α -LA against AMA were

Fig. 7 α -LA reduces the release of cytochrome C and the expression of cleaved caspase-3. Osteoblasts (5×10^6) were preincubated with α -LA for 12 h before treatment with 50 μ M antimycin A (AMA) for 24 h. Osteoblasts were preincubated with α -LA (100 μ M) for 12 h before treatment with 50 μ M antimycin A (AMA) for 24 h. **a** Representative images of western blot analysis and quantitative analysis for cytochrome

C in the cytosolic fractions. COX-4 was used as a control for the fractionation efficiency. **b** Representative images of western blot analysis and quantitative analysis for cleaved caspase-3 (* $P < 0.01$ vs. control group; # $P < 0.01$ vs. AMA treated group; experiments were repeated for four times)

tested by measuring ROS and mitochondrial superoxide generation. AMA-induced ROS and mitochondrial superoxide formation were significantly reduced with α -LA treatment, suggesting that α -LA is highly effective for reducing downstream oxidant production and preventing cell injury. α -LA has been considered as a universal antioxidant therapeutic agent. Over the past decades, it has received considerable attention as a general dietary supplement (Ziegler et al. 1999). Importantly, lipoic acid has been reported to be able to prevent the development of steroid-induced osteonecrosis in rabbits. Inhibited oxidative stress and amendment of vascular endothelial dysfunction is a possible mechanism for this effect (Lu and Li 2012). In addition, Aydin and colleagues reported that α -LA supplementation promotes healing of femoral fractures in rats (Aydin et al. 2014).

PI3K and CREB have been shown to play an essential role in osteoblast-like cell proliferation and differentiation (Carpio et al. 2001). In the present study, pretreatment of MC3T3-E1 cells with α -LA increased PI3K activity and CREB phosphorylation inhibited by AMA. Inactivation of PI3K directly or indirectly affects CREB phosphorylation and activation (Vivanco and Sawyers 2002). CREB is imported into the mitochondrial matrix and, when phosphorylated by protein kinases, promotes the synthesis of mitochondrially encoded subunits of oxidative phosphorylation complexes (De Rasmio et al. 2009). However, the pathological mechanism of osteoporosis is complex. α -LA may be an antioxidant that it is not clear that this effect is the mechanism by which protection was conferred.

Taken together, our results indicated that α -LA can significantly decrease AMA-induced cell damage via improving mitochondrial function, activating the PI3K and CREB pathways, and inhibiting apoptosis. As α -LA is an antioxidant, we speculate that it might not be a direct mechanism of α -LA action since it is not acting directly on the point of AMA's action. Similarly, improvement of mineralization, increased survival, and mitochondrial function appear to be secondary to α -LA's effect on AMA's action. The precise mechanism still requires a further in vivo study in the future.

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