The Antioxidant and Pro-oxidant Impacts of Varying Levels of Alpha-Lipoic Acid on Biomarkers of Myoglobin Oxidation in Vitro

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ABSTRACT

R-alpha-lipoic acid (R-ALA) has been known to protect protein oxidation and lessen the pathogenesis of oxidative-related multiple diseases; however, its dosing remains unresolved. This study aimed to examine whether in vitro R-ALA varying levels would have antioxidant or pro-oxidant impacts on biomarkers of myoglobin oxidation in terms of carbonyls and free thiols for myoglobin upon long-term incubation. Myoglobin (1mg/mL) was concentrated with 6 different concentrations of R-ALA: 50 µM, 100 µM, 500 µM, 1mM, 2mM and 4mM for 30 days at pH 6.6 and temperature 37 °C. Myoglobin oxidative modifications as protein carbonyls and its oxidative defense as free thiols were determined by standard procedures. Thirty-day coincubation of native myoglobin with R-ALA at 500 µM, 1mM, 2mM, and 4mM significantly (p<0.05) elevated carbonyls (2.51±0.19; 2.59±0.22; 2.71±0.32 and 2.79±0.39 nmol/ mg protein respectively) compared to their levels in native control myoglobin (1.67±0.43 nmol/ mg protein) and significantly (p<0.05) decreased free thiols (4.60±0.36; 4.49±0.46; 4.38±0.28 and 4.07±0.39 nmol/ mg protein respectively) against their levels in native control myoglobin (5.71±0.62 nmol/ mg protein). Conversely, coincubation of myoglobin with 50µM and 100µM R-ALA reduced carbonyls (1.02±0.29 and 0.9±0.19 nmol/ mg protein respectively) compared to the control levels (1.67±0.43 nmol/ mg protein) and elevated free thiols (6.1±0.28 and 6.83±0.28 nmol/ mg protein respectively) against control levels (5.71±0.62 nmol/ mg protein) levels; 100µM elicited significant (p<0.05) differences, but 50µM did not. Findings indicate that high levels of R-ALA (0.5-4mM) provoked myoglobin oxidative damage while moderate levels (50-100µM) protected protein upon any spontaneous oxidative damage during long-term coincubation. Thus, R-ALA concentrations, which set the balance between R-ALA pro- and antioxidants, dictate the primary impacts of R-ALA on myoglobin redox status. Additional in vivo investigations are needed to assess the therapeutic insights of current findings.

Keywords: Antioxidants; pro-oxidant; Lipoic acid; Myoglobin; Protein carbonyls; Protein-free thiols; Chronic diseases

INTRODUCTION

Protein oxidation has often been associated with the dysfunction of human proteins, which contributes significantly to the pathogenesis of multiple metabolic diseases (Ahmad et al., 2020). The oxidative modification of protein can be provoked by reactive species directly or indirectly by the adduction with oxidative stress secondary
products (Hawkins & Davies, 2019). Among the various oxidative modulations of protein, carbonyls generation, and loss of its free thiols’ moiety are routinely used as a biomarker for oxidation-dependent protein damage (Weber et al., 2015; Hawkins & Davies, 2019).

Protein carbonyls generation is a crucial oxidative mechanism resulting in structural modification of human proteins mediated by oxidative stress (Fedorova et al., 2015). Amino side residues of the protein react with oxidative stress secondary products or can be directly oxidized by reactive species yielding carbonyl intermediates (Hawkins & Davies, 2019). Protein carbonyls generation is one of the early indicators for the estimation of the extent of protein oxidative damage (Weber et al., 2015).

It has readily been found that reactive species induce oxidation of protein thiols, thus attenuating protein antioxidant defense by the elimination of reactive sulfhydryl groups (Poole, 2015). The thiols group of protein is strongly susceptible to oxidative attack giving rise to the generation of sulfur-centered radicals and disulfide bonds (Poole, 2015; Baba & Bhatnagar, 2018) as well as would also lead to intra- and intermolecular cross-links in proteins (Fra et al., 2017).

The increased demand for energy in the cardiac and skeletal muscle raises the possibility of free radical-mediated damage (Serra et al., 2018). Myoglobin, a hemoprotein in the cardiac and skeletal muscle, essentially maintains the oxygen homeostasis in tissues (Wittenberg, 2009). It also regulates reactive species as well as nitric oxide levels in the cardiomyocytes (Hendgen-Cotta et al., 2014). Thereby, it is a critical factor affecting the redox reaction cascade in the cardiac muscle, which helps metabolic and functional protection of the heart from oxidative damage (Wittenberg, 2009; Hendgen-Cotta et al., 2014; Serra et al., 2018). Because of its high susceptibility to oxidative changes, human myoglobin has been considered as one of the main physiological targets for many in vitro studies of oxidative damage (Roy et al., 2004 & 2010; Ghelani et al., 2018).

R-alpha-lipoic acid (R-ALA) is the naturally occurring biologically active dithiol found in foods of animal origin (Shay et al., 2009). It is well documented that LA is a potent biological antioxidant exploiting the reactivity of nucleophilic thiol groups. Several recent studies demonstrated that LA cotreatment reduces oxidative stress as well as regenerates reduced forms of other antioxidants (Aldini et al., 2013; Zhang et al., 2017; Andreeva-Gateva et al., 2020). The fact that the universal protective impacts of ALA are owing to absolutely its antioxidant proprieties has been interrogated. Many investigators supported the idea that ALA is potentially able to induce oxidation of proteins and thus impair their biological functions (Scott et al., 1994; Slepneva et al., 1995; Dieter et al., 2002; Cakatay & Kayali, 2005; Cakatay et al., 2005; Kayali et al., 2006). This controversy may reflect differences in research protocols and procedures, particularly ALA forms and concentrations, and incubation time and conditions (Scott et al., 1994; Slepneva et al., 1995; Dieter et al., 2002; Cakatay & Kayali, 2005; Cakatay et al., 2005; Kayali et al., 2006; Aldini et al., 2013; Zhang et al., 2017; Andreeva-Gateva et al., 2020). Thus, we hypothesized that the concentrations of R-ALA, which set the balance between its pro-and antioxidants, dictate the primary impact of R-ALA on myoglobin redox status. In the present study, we examined whether R-ALA dosing would have antioxidant or prooxidant impacts on protein oxidative damage in native myoglobin upon long-term incubation in vitro.

**MATERIALS AND METHODS**

**Chemicals:** Myoglobin, (R)-(+)–α-Lipoic acid (98%), dinitrophenylhydrazine 97%, and guanidine hydrochloride were purchased from Sigma-Aldrich Co. (St. Louis, Missouri, USA). Trichloro acetic acid ≥ 99.0%, 5,5'-dithiol-bis-(2-nitro benzoic acid) ≥ 98.0% (Ellman's reagent), L-cysteine ≥ 98.0%, dimethyl sulfoxide ≥ 99.5%, dipotassium phosphate, and potassium dihydrogen
phosphate were purchased from Fisher Scientific Inc (Pittsburgh, PA, USA). The Rest of the reagents and solvents used in the current study were of analytical grade, and all solutions were filtered and sterilized before use through a 0.22 μM membrane filter. In all the analytical procedures conducted in this study, each control and/or treatment reaction was replicated 3 times. All the experiments were approved by the University of Jordan Ethical Committee and carried out at the Biochemistry laboratories of the Department of Nutrition, University of Jordan, and the Faculty of Pharmacy, Al-Zaytoonah University of Jordan, Amman, Jordan.

**Protein treatment:** Myoglobin cotreatment was performed in vitro according to the methods previously described by Roy et al (2004 & 2010) as follows: reaction included only 500 µL myoglobin protein (1mg/ml) was incubated in 50 mM phosphate buffer saline (pH 6.6), as native control samples; reaction included 500 µL of myoglobin (1mg/ml) in 50 mM phosphate buffer saline (pH 6.6) pretreated with 100 µL R-ALA dissolved in dimethyl sulfoxide at final concentrations of 50 µM, 100 µM, 500 µM, 1mM, 2mM, and 4mM as treatment samples. All reaction mixtures were prepared in triplicates and incubated in a capped tube in the incubator (Thermo scientific incubator, Waltham, MA, USA) for 30 days at 37°C. At the end of the incubation period, samples subsequently were assayed for protein carbonyls content and protein thiols level, as described below.

**Determination of protein carbonyls:** Protein carbonyls generation is one of the early indicators for the estimation of the extent of protein oxidative damage (Weber et al., 2015). The number of protein carbonyls, as an index of myoglobin oxidative damage in the reaction sample, was determined according to a previous method reported by Levine et al (1994). In brief, 200µL of the reaction sample was incubated with 10 mM 2,4-dinitrophenylhydrazine in 2.5 M HCl at room temperature for 60 minutes; afterward, it was precipitated by 30% (w/v) trichloroacetic acid, left in ice for 5 minutes, and centrifuged in a refrigerated centrifuge (Hettich EBA 12 R, NA, USA) at 15,000 rpm for 4 minutes. The final pellet was washed 3 times with 1 mL 1:1 (v:v) of ethanol-ethyl acetate mixture, then resuspended in 1 mL of 6 M guanidine hydrochloride in 20 mM phosphate buffer saline (pH 6.6), and incubated for 15 minutes at 37°C. Following centrifugation at 15,000 rpm for 4 minutes, the absorbance of the supernatant was recorded spectrophotometrically (UV-1800-UV-VIS, Shimadzu, Japan) at 375 nm and the concentration of carbonyls was calculated based on the absorption coefficient of 2,4-dinitrophenylhydrazine (22,000 M-1 cm-1) and expressed as nmol/mg protein.

**Determination of protein thiols:** The amount of the free thiol group as a measure for the degree of myoglobin's oxidative defense in the reaction sample was assessed according to Ellman's (1959) method. In brief, 70 µL from each reaction solution was mixed with 130 µL of 5 mM 5,5'-dithio-bis-(2-nitrobenzoic acid) in 100 mM phosphate buffer saline (pH 6.6) at room temperature. After 15 min of incubation, the absorbance was recorded spectrophotometrically (UV-1800-UV-VIS, Shimadzu, Japan) at 412 nm against the blank, and the level of protein sulfhydryl group was calculated using L-cysteine's standard curve and expressed as nmol/mg protein.

**Statistical analysis**

Data analysis was performed using statistical analysis software (SAS version 9.4, USA). Statistical significance was assessed by one-way ANOVA followed by Dunnett’s multiple comparison test and Graph Pad Prism-Version 7.0 (GraphPad Software Inc., La Jolla, California USA). Data were expressed as means ±standard deviation. Significance was set at p<0.05.

**RESULTS**

The impacts of R-ALA treatment at various concentrations (50µM-4mM) on the myoglobin carbonyls and free thiols level for 30 days are presented in table 1. Myoglobin oxidative damage was significantly (p<0.05) enhanced in the sample units individually cotreated with 500 µM, 1mM, 2mM, and 4mM R-ALA for 30 days, as
reflected by elevated myoglobin carbonyls content in those treatment mixtures in comparison with their respective control, suggesting a pro-oxidant response to a high level of R-ALA. Conversely, long term coincubation for 30 days of native myoglobin with R-ALA at 50 and 100 µM reduced myoglobin carbonyls content (1.02±0.29 and 0.90±0.19 nmol/ g protein respectively) against native control myoglobin (1.67±0.43 nmol/ g protein), indicating that myoglobin protein could be protected by the low level of R-ALA upon any spontaneous protein oxidative damage. It is worth mentioning that 100µM R-ALA elicited a significant reduction (p< 0.03) in carbonyls level while R-ALA at 50µM failed to reach statistical significance (p< 0.08) in comparison with its respective native control at the end of the incubation period (Figure 1).

**Table 1.** Biomarkers of myoglobin oxidation upon long-term (30 days) cotreatment with different concentrations (50 µM-4 mM) of (R)-alpha-lipoic acid.

| Experimental group          | Protein carbonyls (nmol/mg protein) | Free protein thiols (nmol/mg protein) |
|-----------------------------|-------------------------------------|--------------------------------------|
| Myoglobin native control    | 1.67±0.43                           | 5.71±0.62                            |
| Myoglobin + R-ALA (50 µM)   | 1.02±0.29                           | 6.10±0.28                            |
| Myoglobin + R-ALA (100 µM)  | 0.90±0.19*                          | 6.83±0.28*                           |
| Myoglobin + R-ALA (500 µM)  | 2.51±0.19*                          | 4.60±0.36*                           |
| Myoglobin + R-ALA (1mM)     | 2.59±0.22*                          | 4.49±0.46*                           |
| Myoglobin + R-LA (2 mM)     | 2.71±0.32**                         | 4.38±0.28**                          |
| Myoglobin + R-LA (4 mM)     | 2.79±0.39**                         | 4.07±0.39**                          |

Data are expressed as means ±SD. *P<0.05; **P<0.01; ***P<0.001; (R)-alpha-lipoic acid= R-ALA
Figure 1. Protein carbonyls level (nmol/mg protein) in native myoglobin (Myb) cotreated with various concentrations (50 µM-4 mM) of (R)-alpha-lipoic acid (R-ALA) for thirty days. Data are expressed as means ±SD. *P<0.05; **P<0.01.

The effect of myoglobin cotreatment with six different concentrations of R-ALA for 30 days on the level of free myoglobin thiols is further demonstrated in figure 2. Thirty-day coincubation of native myoglobin with R-ALA at 50 and 100 µM elevated myoglobin free thiols level (6.10±0.28 and 6.83±0.28 nmol/ g protein respectively) against native control myoglobin (5.71±0.62 nmol/ g protein). R-ALA at 100 µM elicited a significant (p<0.02) increment, while R-ALA at 50 µM did not. On the other hand, cotreatment of native myoglobin with 500 µM R-ALA for 30 days significantly (p<0.02) decreased myoglobin free thiols content around 1.2-fold lower than that of native myoglobin (4.60±0.36 vs. 5.71±0.62 nmol/ g protein), and increasing concentration of R-ALA to 4 mM dramatically (p<0.001) diminished myoglobin free thiols level about 1.4-fold lower than that of native myoglobin (4.06±0.39 vs. 5.71±0.62 nmol/ g protein).
DISCUSSION

The present study reveals that long term coincubation of native myoglobin with moderate levels of R-ALA (50-100 µM) induces lower carbonyls and higher free thiols levels than those of the control myoglobin, indicating that human proteins could be potentially protected by such levels of R-ALA upon any spontaneous protein oxidative damage. Consistently, some researchers demonstrated that the cotreatment of myoglobin with LA diminishes protein oxidative stress and regenerates reduced forms of other antioxidants (Zhang et al., 2017; Aydin et al., 2019). Several possible LA-mediated antioxidant mechanisms have been suggested. Antioxidant properties of LA have been attributed to its ability to inhibit reactive species from being generated or scavenge them before they damage cells (Shay et al., 2009; Moura et al., 2015; Salehi et al., 2019). The pair of sulfur atoms in the dithiolane ring of LA attain a distinctive chemical reactivity with an extremely high electron density (Moura et al., 2015). Moreover, LA is likely to alleviate metal-mediated oxidative stress, thus taking advantage of the flexibility of the backbone structure with the charging arrangement as a critical component to partially enclosing metal ions (Tibullo et al., 2017). In this context, it was not determined that any of these R-ALA activities may underlie or represent the mechanistic basis for the inhibition of protein oxidation under the conditions of the current study.

Aside from the ALA antioxidant-related findings, the current study also reveals that high levels of R-ALA (0.5mM-4mM) provoke myoglobin oxidative damage during the long-term coincubation period, as reflected by remarkably elevated protein carbonyls levels and decreased free thiols compared with control myoglobin, suggesting a pro-oxidant response to high R-ALA levels. These findings are in overall agreement with those achieved by Slepneva et al (1995) who displayed that 500 µM LA inactivates microsomal cytochrome P450 reductase purified from rat hepatic microsomes, presumably by directly oxidizing its sulfhydryl groups; the large increase in disulfide bond as LA could react with the thiol group of protein through thiol-disulfide exchange reaction, thus attenuating protein reducing activity by eliminating reactive sulphydryl groups. The thiol group of protein is strongly susceptible to oxidative attack giving
rise to the generation of sulfur-centered radicals and disulfide bonds (Poole, 2015; Baba & Bhatnagar, 2018) and would also lead to intra- and intermolecular cross-links in proteins (Fra et al., 2017). In a series of trials in aged rats supplemented with a high chronic dose of ALA (100 mg/kg), twice daily for two weeks; it has been revealed that such dose enhanced myocardial (Cakatay et al., 2005), brain (Kayali et al., 2006), and plasma (Cakatay & Kayali, 2005) protein oxidative damage markers, as reflected by increased levels of protein carbonyls, nitrotyrosine, and protein thiols.

It is worth stating that the dose-dependent response of the ALA supplementation on protein oxidation was not reported in any of the above-mentioned studies. Thus, the large increase of the dithiolane ring in LA is a critical element that acts as a potent oxidizing agent by itself without the need for the generation of cellular reactive oxygen species (Dicter et al., 2002). Direct oxidative attack on amino acid residues of protein eventually gives rise to carbonyl derivatives (Fedorova et al., 2014). In an intensive study, LA (0.5 mM) effectively reacted with myoglobin at a high oxidation state and generated nonfunctional sulfmyoglobin as contrasted with cysteine and glutathione (Romero et al., 1992). LA at a fairly high level (500 µM), has been reported to produce reactive sulfur-centered radicals that can impair biological molecules like creatine kinase and α1-antiproteinase proteins in human plasma (Scott et al., 1994). Furthermore, it has been reported that LA is optimal for catalyzing intramolecular disulfide bonds generation in human proteins that exploit their high biochemical activity with redox-susceptible two-thiol groups as a critical component, thereby reducing protein antioxidant defense (Cakatay, 2006). Although the pro-oxidant activity of LA is well documented, the underlying biochemical machinery remains unclear.

To our knowledge, the current study is the first investigation that examined the potential dose-dependent impact of specifically R isomeric form of ALA against oxidative myoglobin damage. The majority of ALA–related biological studies did not well define the form of LA that has been used (Gomes & Negrato, 2014). It is known that the isomeric R-ALA is the naturally occurring biologically active form, whereas the S-ALA is the synthetic inactive form (Shay et al., 2009; Salehi et al., 2019). Thus, this study presents typical data relevant to the R-ALA and its influence on myoglobin redox status.

Although the impact of R-ALA on human proteins has been formerly tested counting the oxidative-mediated damage, a wide range of experimental ALA doses were used among former in vitro and in vivo studies, the majority of these doses were high enough to be far from physiological relevant conditions (Suzuki et al., 1992 & 2017; Ghelani et al., 2018). The ability of R-ALA at a low level (50–100 µM) as those implemented in the present research to inhibit protein oxidative damage has merely been assessed in the literature. Suzuki et al. coincubated bovine serum albumin with 20 mM lipoate (Suzuki et al., 1992). A study recorded a dose-dependent inhibition of glycation-provoked oxidative damage using an in vitro myoglobin glycation model cotreated with different ALA doses from 1 up to 4 mM (Ghelani et al., 2018). Whereas the experimental conditions of such studies, particularly the reactant concentrations, were substantially far from physiological levels, the present research has recognized that such high concentrations of LA dosing markedly enhanced myoglobin oxidative damage and dramatically diminished oxidative defense of proteins.

The overall findings of this research indicate that the deleterious impacts of R-ALA on the myoglobin redox status could be supposed to be dose-dependent since it has antioxidant as well as prooxidant activities. In the light of a wide range of daily supplemented doses of ALA (up to 2400 mg/day) have been reported in human trials (Shay et al., 2009), and the peak plasma concentrations of ALA was detected to reach a level of 100 μM up to 200 µM after intravenous supplementation of 600 mg ALA among diabetic patients (Moini et al., 2002), and could also reach 0.0194 mM peak plasma concentration after a common oral dose (Gomes & Negrato, 2014). Thus, an appropriate dose selection of ALA that matches the physiological relevant level is crucial.
CONCLUSIONS

Taken together, this in vitro study presents indications that R-ALA is a bifunctional compound that may damage or protect biological molecules. The results emphasize that R-ALA nutritional supplements should be utilized with caution to prevent potential adverse impacts associated with pro-oxidant bioactivities. Henceforth, current findings focus on the urgency of the dose monitoring of ALA supplementation to present innovative information associated with the ALA's optimal dose that could be fulfilled by oral dosing in clinical settings. Further research is thereby justified concerning the typical pharmacological dose of ALA and its safety.

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التأثيرات المضادة والمؤيدة للأكسدة لمستويات مختلفة من حامض ألفا ليبوك، على الميغوفيسبين في المختبر

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ملخص
إنّ من المعروف أن حامض الليبوكيك يحمل أكاسدة البروتين ووقت من تطور الأمراض المتعددة المرتبطة بالأكسدة، ومع ذلك، تبقى جرعاتهاات تحدّد، ولهذا هدف هذه الدراسة هو حاصل إذا كانت تأثير حامض الليبوكيك في معدلاته كانت تأثيرات مضادة أو مؤيدة للأكسدة على الميغوفيسبين من حيث كميات البروتين والثيول الحرة عن حضانة على المدى الطويل، وكذلك معالجة الميغوفيسبين (1 ملغم/ملي مول) في المختبر بستة تراكيز مختلفة من حامض الليبوكيك (50 ميغوفيسبين، 100 ميغوفيسبين، 500 ميغوفيسبين، 1 ملي مول، 2 ملي مول، 4 ملي مول) لمدة 30 يوماً عند درجة المحمولة 6.6 درجة الحرارة 37 درجة مئوية. وتم قياس التغيرات الأكسدة في الميغوفيسبين من خلال قياس تركيز كاربنيلات البروتين ودفاغها التأكسدة كثائيات الحرة بطرق القياسية للتحليل، وأيضاً استخراج الميغوفيسبين الأكسدة لمدة 30 يومًا مشابهًا (P<0.05) للوجود الفاكهة في الزيتون (2.51 ± 0.19 و 2.59 ± 0.22 و 2.71 ± 0.32 و 0.39 نانو مول/ملي مول)، في مختبر كاربنيلات البروتين (1.67 ± 0.43 نانو مول/ مليم بروتين)، وتؤتي إلى انخفاض معنوي (P<0.05) في مستويات البروتينات الحرة (0.56 ± 0.46 و 0.49 ± 0.28 و 4.38 ± 0.07 نانو مول/ملي مول بروتين). وأيضاً تتراهي بين مستويات البروتينات الحرة (0.62 ± 5.71 و 0.62 ± 5.71 نانو مول/ملي مول بروتين)، وعلى الأكسدة، يمكن أن تؤتي إلى السعودية في الميغوفيسبين الأكسدة الشاهد (1.67 ± 0.43 نانو مول/ملي مول بروتين)، ورافع في ميغوفيسبين الأكسدة الشاهد (0.62 ± 5.71 نانو مول/ملي مول بروتين)، بالإضافة إلى الوصول إلى أن التركيز المرتفع (0.5- 4.0 ملي مول) من حامض الليبوكيك يقلص تركيز كاربنيلات البروتين، بينما التأكسد المستخدم من (50 – 100 ميغوفيسبين) أدى إلى زيادة من الأكسدة خلال احتضانه على المدى الطويل، وعلى النقيض، تبين التأكسد الأولية لحامض الليبوكيك على حالة أكاسدة الميغوفيسبين أن هناك حاجة إلى مزيد من الدراسات على مستوى الجسم الحي لتقدير الرؤى الالعاجية لنتائج هذه الدراسة.

الكلمات الدالة: مضادات الأكسدة، مؤيدات الأكسدة، حامض الليبوكيك، الميغوفيسبين، كاربنيلات البروتين، الثيول الحرة، الأمراض المزمنة.