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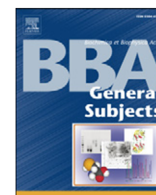

Alpha-lipoic acid supplementation protects enzymes from damage by nitrosative and oxidative stress

Sylvia Hiller [a], Robert DeKroon [b], Eric D. Hamlett [c], Longquan Xu [a], Cristina Osorio [d], Jennifer Robinette [c,d], Witold Winnik [e], Stephen Simington [b], Nobuyo Maeda [a], Oscar Alzate [f,⁎⁎], Xianwen Yi [a,⁎]

[a] Department of Pathology and Laboratory Medicine, The University of North Carolina at Chapel Hill, Chapel Hill, NC, United States
[b] Lineberger Comprehensive Cancer Center, The University of North Carolina at Chapel Hill, Chapel Hill, NC, United States
[c] Department of Neurosciences, Medical University of South Carolina, Charleston, SC, United States
[d] Systems Proteomics Center, The University of North Carolina at Chapel Hill, Chapel Hill, NC, United States
[e] Proteomic Research Core Unit, NHEERL, U.S. Environmental Protection Agency, Research Triangle Park, NC, United States
[f] Texas A&M Health Science Center, College Station, TX, United States

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A B S T R A C T

Background: *S*-nitrosylation of mitochondrial enzymes involved in energy transfer under nitrosative stress may result in ATP deficiency. We investigated whether α-lipoic acid, a powerful antioxidant, could alleviate nitrosative stress by regulating *S*-nitrosylation, which could result in retaining the mitochondrial enzyme activity.
Methods: In this study, we have identified the *S*-nitrosylated forms of subunit 1 of dihydrolipoyllysine succinyltransferase (complex III), and subunit 2 of the α-ketoglutarate dehydrogenase complex by implementing a fluorescence-based differential quantitative proteomics method.
Results: We found that the activities of these two mitochondrial enzymes were partially but reversibly inhibited by *S*-nitrosylation in cultured endothelial cells, and that their activities were partially restored by supplementation of α-lipoic acid. We show that protein *S*-nitrosylation affects the activity of mitochondrial enzymes that are central to energy supply, and that α-lipoic acid protects mitochondrial enzymes by altering *S*-nitrosylation levels.
Conclusions: Inhibiting protein *S*-nitrosylation with α-lipoic acid seems to be a protective mechanism against nitrosative stress.
General significance: Identification and characterization of these new protein targets should contribute to expanding the therapeutic power of α-lipoic acid and to a better understanding of the underlying antioxidant mechanisms.

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1. Introduction

Nitric oxide (NO) is a soluble and highly diffusible gas generated by a wide variety of cell types including vascular endothelial cells. NO plays an important role in endothelium-derived smooth muscle relaxation [1]. Excess production of endogenous NO under inflammatory conditions can be toxic to vascular endothelial cells [2]. Unregulated NO production leads to an increase in Reactive Nitrogen Species (RNS) resulting in nitrosative stress (NS), which may give rise to significant pathological outcomes including cellular injury and disease [3]. Specifically, NS is involved in the pathogenesis of low-grade chronic inflammatory-associated diseases such as atherosclerosis and type 2 diabetes [4,5]. A better understanding of NS-associated underlying mechanisms may help improve therapeutic outcomes for these diseases.

Evidence shows that NO regulates protein function via *S*-nitrosylation, a reversible redox post-translational modification that adds an NO group to a reactive cysteine residue in regulatory proteins [6]. In recent years, protein *S*-nitrosylation has received much attention as an ubiquitous regulatory strategy for multiple biological processes [7,8,9]. NO-induced protein *S*-nitrosylation results in the modulation of active-site thiol and disulfide formation that could either inhibit or enhance the catalytic activity of redox-sensitive enzymes [10]. Some investigations have suggested that *S*-nitrosylation is involved in both normal and abnormal vascular function [6], including regulation of key metabolic pathways [11].

Endothelial cells form the inner cellular lining of blood vessels and play a key role in regulating vascular cellular homeostasis, vascular integrity, vasorelaxation, and local inflammation [12]. Dysfunction of the endothelium may result in pathogenic processes associated with a number of cardiovascular diseases such as atherosclerosis, hypertension, heart attack and stroke [13]. Because the endothelium constitutively expresses endothelial nitric oxide synthase (eNOS) and is exposed to NO

⁎ Correspondence to: X. Yi, Department of Pathology and Laboratory Medicine, The University of North Carolina at Chapel Hill, 710 Brinkhous-Bullitt Building, Chapel Hill, NC 27599-7525, United States.
⁎⁎ Correspondence to: O. Alzate, Texas A&M Health Science Center, ILSB 1155, 301 Old Main Dr., College Station, TX 77845, United States.
E-mail addresses: alzate@medicine.tamhsc.edu (O. Alzate), xyi2000@med.unc.edu (X. Yi).

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derived from the inducible nitric oxide synthase (iNOS) isoform under inflammatory conditions [14]. NO increase has been found to coincide with an increase in protein *S*-nitrosylation, so it is an ideal tissue for studying *S*-nitrosylation-dependent signals [15]. It is thought that the endogenous production of NO in the endothelium is not sufficient to inhibit endothelial cell activation during inflammation, and that higher concentrations of NO derived from the use of an exogenous NO donor, DETA-NONOate, is required for inducing higher levels of protein *S*-nitrosylation [16].

α-Lipoic acid (1,2-dithiolane-3-pentanoic acid; LA) is a di-thiol antioxidant with diverse functions such as RNS quenching, anti-inflammatory activity [17], and may be therapeutic in mitochondria-related disorders [18,19]. LA is also a cofactor for several enzymes including pyruvate dehydrogenase complex (PDC) and α-ketoglutarate dehydrogenase complex (KDC), two mitochondrial enzymes involved in glucose metabolism and energy production. In a previous study, we reported that endogenous LA production is necessary for mouse embryo survival [20]. This result led us to propose that in addition to its antioxidant capacity, LA is essential for the maintenance of normal levels of energy metabolism [20]. We also found that LA increased the mitochondrial antioxidant capacity, and partially restored mitochondrial enzyme activity and increased ATP yield during sepsis in which an excess of NO had been produced [21]. Other investigators have also reported that the exposure of endothelial cells to small molecular *S*-nitrosothiols significantly down-regulates their mitochondrial function [22]. Therefore, we hypothesized that inflammation-induced excess NO production increases protein *S*-nitrosylation, which may result in diminished oxidative phosphorylation and decreased energy production in some mitochondrial enzymes. We propose that LA is able to alleviate mitochondrial dysfunction by regulating protein *S*-nitrosylation, thus helping cells to retain their energy supply. In this study, we found that through alteration of protein *S*-nitrosylation, LA partially restores mitochondrial oxidative phosphorylation reserve capacity and ATP production. These findings shed light on a novel mechanism by which LA is able to rescue energy deficiency in inflammation-associated diseases, including low-grade chronic inflammation diseases, such as atherosclerosis and type 2 diabetes.

2. Materials and methods

All animal protocols were reviewed and approved by the Ethics Committee of the University of North Carolina at Chapel Hill (Protocol number: 13-208-0).

2.1. Reagents

(Z)-1-[2-(2-aminoethyl)-*N*-(2-ammonioethyl) amino] diazen-1-ium-1,2-diolate (DETA-NONOate) was obtained from Cayman Chemical (Ann Arbor, MI). Protease inhibitor cocktail (Complete™ Mini EDTA-free protease inhibitor cocktail) was from Roche Applied Science (Indianapolis, IN). Antimycin A, coenzyme A, cytochrome C, ferricytochrome C, carbonylcyanide *p*-triflouromethoxyphenyl-hydrazone (FCCP), oligomycin, potassium cyanide, *S*-methyl methanethiosulfonate (MMTS), rotenone, thiamine pyrophosphate, urea, CHAPS, neocuproine and cuprizone were all from Sigma-Aldrich (St. Louis, MO). Decylubiquinol was from Santa Cruz **Biotechnology, Inc.** (Dallas, TX); and R(+) α-lipoic acid (LA) was from Toronto Chemical Research (Toronto, Canada). NHS-Cy dyes and dye maleimides were from GE Healthcare (Piscataway, NJ).

2.2. Cell cultures

Primary aortic endothelial cells from C57BL/6J mice (Cell Biologics, Chicago, IL) and mouse brain endothelioma cells (bEnd.3, American Type Culture Collection, Manassas, VA) were used in these studies. The latter, an alternative source of endothelial cells, was used to compare the possible different responses to NS between the primary cells and the cell line. The primary cells and the brain cells were cultured in Complete Mouse Endothelial Cell Medium (Cell Biologics), and in Dulbecco's Modified Eagle Medium (DMEM) containing 10% fetal bovine serum (Gibco, Langley, OK), respectively. Both cell types were maintained in a humidified incubator gassed with 5% CO_2, 95% air at 37 °C. LA was dissolved in 100 mM phosphate buffer (pH 7.2) after being converted into LA sodium salt. A concentration of 50 μM of LA shows effective protection against oxidative stress in cell cultures [23–25]. Preparation of DETA-NONOate was based on the method by Dranka et al. [26], in which an appropriate dose to induce NS in endothelial cells (~250 μM) was established. The amount of NO from DETA-NONOate was released at a constant rate (~150 nM/min) during the first 8 h, and was monitored by absorbance spectroscopy. This concentration of DETA-NONOate is capable of decreasing the oxygen consumption rate (OCR) [26]. Therefore, this is the concentration that we used for cell treatments in this study. Cell counting was performed using a Neubauer chamber, and the number of dead cells was determined by trypan-blue exclusion before and after treatment. The analysis was performed with 4 groups, 5 samples per group, which were created as follows from cell cultures grown to ~60% confluence: group 1: untreated cells (control group); group 2: cells in regular medium for 6 h followed by treatment with LA for 12 h (LA group); group 3: cells treated with DETA-NONOate for 6 h from time zero and then changed to fresh medium for 12 h (NO group); and group 4: cells treated with DETA-NONOate for 6 h, followed by a change to an LA-containing medium for 12 h (NO + LA group).

2.3. Mitochondrial isolation and purity examination

Mitochondria were isolated from aortic endothelial cells using a Mitochondria Isolation Kit for cultured cells, the reagent-based method (Thermo Fisher Scientific, Rockford, IL, Product No. 89874) following the manufacturer's instructions. Certain critical points of purification procedure provided by other optimized protocols were applicable to our purification [27,28]. Harvested cells were homogenized in ice-cold isolation buffer (100 mM Tris–MOPS, 10 mM EGTA/Tris, 200 mM sucrose, pH 7.4). The homogenate was centrifuged for 10 min at 3000 ×*g* at 4 °C. The supernatants were collected in isolation buffer, and then centrifuged for 10 min at 10,000 ×*g* at 4 °C. The resulting pellets were re-suspended in ~1 mL isolation buffer, kept on ice and used within 4 h or stored at −80 °C for later use.

The purity of the isolated mitochondria compared to cytosolic fractions was assessed via Western blot analysis for HSP60 or Tubulin and quantitated using Image Studio Light software (LICOR Biotechnologies, Lincoln, Nebraska). Protein concentrations were measured using the Thermo Scientific™ BCA Protein Assay Kit (Product No. 23,225). A total of 20 μg of cytosolic proteins and 18.8 μg of mitochondrial proteins (the different loading amounts were normalized during quantitative calculation afterwards) were loaded on 12% kD™ Mini-PROTEAN® TGX™ Precast gels (BioRad, Hercules, CA) and transferred onto nitrocellulose membranes. Membranes were blocked with 4% BSA for 1 h. Proteins were detected with anti-HSP60 (Rabbit polyclonal, 1:10,000, Abcam Cat# AB46798) and anti-tubulin (Mouse monoclonal anti-tubulin, beta III isoform, Chemicon Cat# MAB 1637), followed by secondary horseradish peroxidase (HRP)-conjugated antibody, either rabbit IgG (1:500) or mouse IgG (1:500), from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA). Signals were detected with a chemiluminescence detection kit (Millipore).

2.4. Determination of mitochondrial levels of antioxidant and oxidative stress

Isolated mitochondria prepared as described above were deproteinated with 5% trichloroacetic acid. The level of reduced glutathione (GSH), a major endogenous antioxidant in mitochondria, was determined with a Glutathione Assay Kit from Cayman Chemical. The protein concentration of each sample was determined using a bicinchoninic acid assay (BCA;

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