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



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ABSTRACT

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

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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*-trifluoromethoxyphenyl-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 Biologicals, 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 Biologicals), 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% CO₂, 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 μ M 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 μ M) 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 \times g at 4 °C. The supernatants were collected in isolation buffer, and then centrifuged for 10 min at 10,000 \times 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 μ g of cytosolic proteins and 18.8 μ g 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 deproteinized 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;

Thermo Fisher Scientific). Mitochondrion oxidative stress was measured using 4-Hydroxynonenal (4-HNE) assay kit in accordance with manufacturer's specifications (Cell Biolabs, Inc. San Diego, CA).

Mitochondrial nitrate/nitrite (NO_x) levels, as the stable metabolic end products of NO, were determined using a colorimetric assay kit (Cayman Chemical). The absorbance was read on a Biotek Synergy HT Multi-Mode microplate reader (Winooski, VT, USA) using a wavelength of 540 nm. Protein carbonyl content was assessed by using an OxiSelect Protein Carbonyl ELISA kit according to the manufacturer's instructions (Cell Biolabs, Inc.).

2.5. Determination of ATP production

ATP generation in the DETA-NONOate-treated primary aortic endothelial cells was analyzed by a luciferase substrate assay (Invitrogen, Carlsbad, CA). Briefly, the cells were homogenized and sonicated in ATP Assay Buffer provided by the company. After centrifugation at 12,000 × g for 5 min at 4 °C, the supernatant was removed and used for ATP measurement in an Orion Microplate Luminometer (Berthold Detection Systems, Titertek Instruments, Inc., Huntsville, AL). The amount of ATP was normalized to the protein concentration as determined by the BCA method.

2.6. Measurement of the oxygen consumption rate (OCR) and the extracellular acidification rate (ECAR)

OCR and ECAR were measured using the XF24 extracellular flux analyzer (Seahorse Bioscience, Billerica, MA) following the manufacturers' instructions. Briefly, primary aortic endothelial cells were seeded at a density of 5 × 10⁴ cells per well, treated with 250 μM DETA-NONOate, followed by 50 μM LA. The cells were washed with PBS and incubated with bicarbonate-free (no buffering capacity) DMEM at 37 °C for 1 h. Measurements were taken sequentially, as the cells were incubated, with the following chemicals: (1) oligomycin (5 μg/mL), a

reversible inhibitor of ATP synthase; (2) FCCP (5 μM), an electron uncoupler of ATP synthesis; and (3) antimycin A (40 μM), a complex III inhibitor. Basal levels of OCR and ECAR were obtained in the cultured cells without the additives.

2.7. Proteomic analysis

Confluent cells were washed four times with cold PBS, re-suspended in 500 μL of cold PBS supplemented with Complete™ protease inhibitor cocktail without EDTA, and spun at 2000 × g for 20 min at 4 °C. Pelleted cells were re-suspended in 500 μL lysis buffer (8 M urea, 4% CHAPS, 30 mM Tris, pH 8.5) supplemented with protease inhibitor cocktail, and then sonicated on ice three times, for 20 s each with 10 s rest. After centrifugation, the resulting supernatants were cleaned up using a 2D Clean-Up kit (GE Healthcare), according to the manufacturer's instructions. The final pellet was re-suspended either in lysis buffer or S-nitrosylation-labeling buffer (8 M urea, 4% CHAPS, 20 mM Tris, pH 7.3) and the protein concentration was determined using a 2D Quant kit (GE Healthcare) as instructed by the manufacturer.

A modified fluorescence-switch method [29] was used to detect S-nitrosylated proteins as follows. Protein samples were re-suspended in S-nitrosylation-labeling buffer, MMTS was added to 100 μg of protein sample to produce a final concentration of 20 mM MMTS, and then incubated at room temperature for 1 h while being protected from light. The samples were cleaned up as previously described [30] to remove unbound MMTS. Then the samples were re-suspended in S-nitrosylation-labeling buffer, and finally ascorbic acid was added to a final concentration of 100 mM (Fig. 1). Samples were cleaned again and the resulting pellets were re-suspended in S-nitrosylation-labeling buffer, followed by the addition of 500 pmol of Cy5-maleimide and flushed with nitrogen to decrease the possibility of protein oxidation. Samples were incubated at room temperature for 2 h followed by incubation overnight at 4 °C, protected from light.

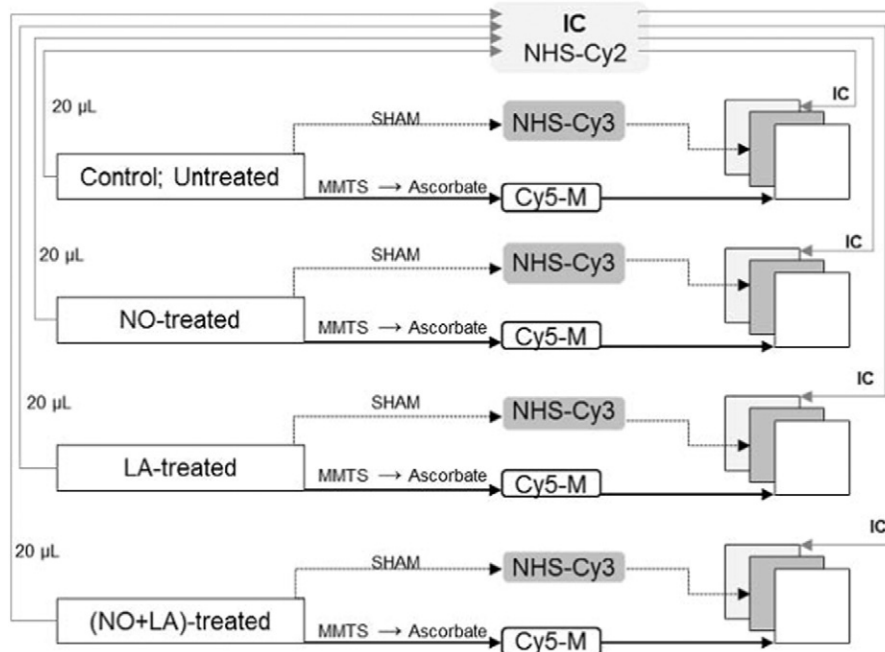


Fig. 1. Experimental design. Protein preparations from four samples were used for SNO analysis: untreated samples used as the Control group; samples from cells treated with nitric oxide (NO group); samples from cells treated with LA (LA group); and samples from cells that were treated with NO, followed by treatment with LA (NO + LA). Each group was divided into three fractions: fraction one consisted of 20 μL from each sample, these samples were pooled, and labeled with NHS-Cy2 to develop an internal control (IC). This IC was used for image normalization, and differential expression analysis; fraction two, consisted of 100 μg of total protein sample that was treated first with MMTS to block free sulfhydryl groups, then treated with ascorbate to remove the NO groups, and then labeled with Cy5-maleimide, to label the free -SH groups that resulted from the ascorbate treatment. This fraction provides a red color when scanned with the Typhoon TRIO +; and fraction three (SHAM) consisting of 100 μg of sample and treated as fraction two, without adding MMTS, or ascorbate. This fraction was labeled with NHS-Cy3, and used to determine the total amount of protein. In principle, it is expected that every protein that was initially nitrosylated would provide a fluorescent red color after the treatment. After sample treatment and labeling, the proteins were fractionated by 2D Fluorescent gel electrophoresis. The differences in total protein expression, and protein nitrosylation were determined with the Decyder 2D software (DeKroon et al., 2011—Ref. 29).

An internal control (IC) was prepared by pooling equal amounts of protein (20 μg) from all control samples, which were labeled with 400 pmol/50 μg protein of NHS-Cy2. Aliquots from the IC were added to each gel. A 100 μg aliquot of each sample was processed as the S-nitrosylation sample without adding either MMTS or ascorbate. After clean-up, these samples were labeled with 400 pmol of NHS-Cy3 to determine total protein expression changes. The labeling reactions were carried out on ice for 30 min, protected from light. To quench the reaction, 1 μL of 10 mM lysine was added, and the reaction was incubated for an additional 10 min on ice in the dark. After labeling, corresponding samples were combined as shown in Fig. 1. An equal volume of 2 \times sample buffer (8 M urea, 4% CHAPS, 20 mg/mL DTT, 2% (v/v) IPG buffer 4–7 (GE Healthcare)) was added and the mixture was placed on ice for 15 min. Rehydration buffer (8 M urea, 4% CHAPS, 2 mg/mL DTT, 1% (v/v) IPG buffer 4–7) was added to a final volume of 250 μL .

2.8. Differential fluorescence 2D gel electrophoresis and image analysis

Differential fluorescence 2D gel electrophoresis was carried out as reported previously [31]. After electrophoresis, the gels were scanned with a Typhoon Trio + (GE Healthcare). Gel images were analyzed as described [31] with Decyder 2D software Ver7.0 (GE Healthcare). The resulting images for S-nitrosylated proteins and total protein differential expression were analyzed separately, using the same internal control because the nitrosylated proteins within each group were assigned unique spot numbers, and the “total” protein spot numbers very often do not correlate to the same protein spot [31] due to the isoelectric point shift resulting from the different labels. Spot maps for each gel image (individual samples and internal control) were matched and an ANOVA (SAS Institute Inc., Cary, NC) comparison was performed between Control, LA, NO, and (NO + LA) for each spot. The extended data analysis (EDA) component of DeCyder 7.0 was used for principal component analysis (PCA) and hierarchical cluster analysis (HCA).

2.9. Protein identification by mass spectrometry (MS)

For protein identification purposes, approximately 400 μg of total protein consisting of equal contributions from each experimental group was

separated by 2D gel electrophoresis for spot picking. Matching protein spots between the pick gel and the analytical gels were picked using an Ettan spot picker (GE Healthcare), and digested with trypsin as previously described [32]. Proteins were identified using a 4800 MALDI TOF/TOF (Applied Biosystems (AB), Foster City, CA) mass spectrometer either at the UNC Proteomics Core Facility or the US EPA NHEERL Proteomics Research Core Facility. Mascot software (<http://www.matrixscience.com/>) was used to identify the proteins from background-subtracted MALDI-MS and MS/MS data. MALDI-MS acquisition was followed by MALDI-MS/MS peptide sequencing, and the final protein identification was obtained using Protein Pilot 3.0 software (AB SCIEX, Framingham, MA), searching against the mouse (*Mus musculus*) sub-database of the SwissProt protein database (<http://expasy.org/sprot/>). Mass-selected MALDI-MS/MS spectra acquisition was performed to increase the protein sequence coverage. The reported proteins were identified based on at least two high-quality (95–99% peptide confidence scores in Protein Pilot) peptide sequence identifications made from the MS/MS data [32].

2.10. Alpha-ketoglutarate dehydrogenase (KDC) and complex III activities in mitochondria

KDC activity in isolated mitochondria was determined by monitoring the reduction of NAD⁺ at 340 nm. Briefly, isolated mitochondria were resuspended in buffer containing 25 mM KH₂PO₄, 5 mM MgCl₂, 0.5 mM EDTA, 0.1% (v/v) Triton X-100, 40 mM rotenone, 1.0 mM NAD⁺, 0.2 mM thiamin pyrophosphate, and 0.1 mM Coenzyme A, pH 7. The mitochondria were sonicated in a water bath at room temperature for 30 s. A reaction mixture containing 5 mM MgCl₂, 40 mM rotenone, 0.1 mM Coenzyme A, 0.2 mM thiamine pyrophosphate and 1 mM NAD⁺ were added to the mitochondrial medium to reach a final concentration of 0.4 mg mitochondrial protein/mL. The reaction was initiated by the addition of 2.5 mM α -ketoglutarate [33]. Complex III (ubiquinol–ferricytochrome C oxidoreductase; EC 1.10.2.2) activity in mitochondria was measured by following the increase in absorbance at 550 nm due to the reduction of ferricytochrome C [34]. The reaction mixture consisted of 25 mM KH₂PO₄, pH 7.4, 5 mM MgCl₂, 2 mM KCN, 2.5 mg bovine serum albumin, 50 μM cytochrome C, and 4 mg rotenone.

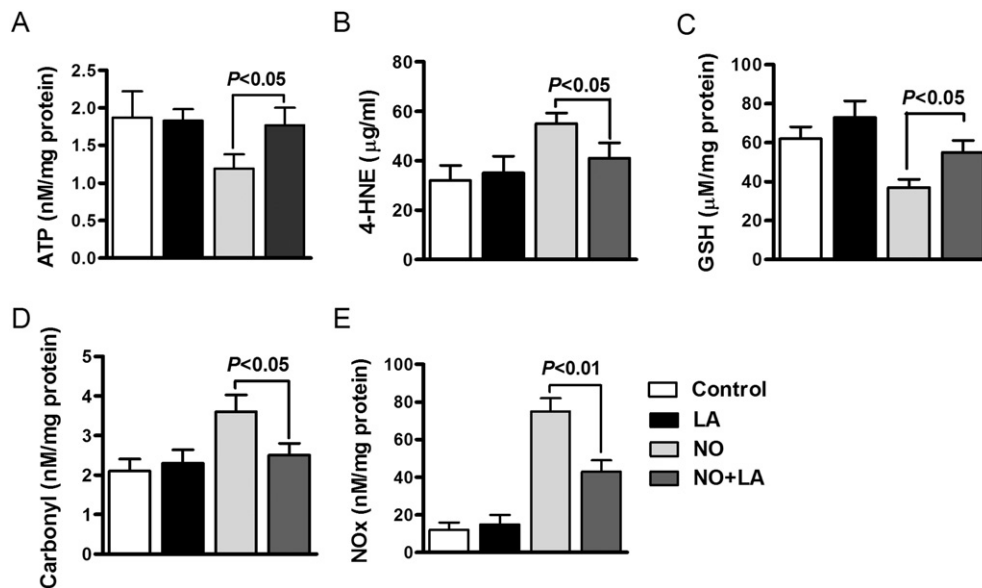


Fig. 2. LA increased ATP production and mitochondrial GSH levels. A) ATP production in primary aortic endothelial cells treated with the NO donor, DETA-NONOate, in the presence or absence of LA. Each group was collected from 3 independent experiments. B) Mitochondrial 4-HNE level was increased after NO treatment and that LA-treated cells significantly offset this effect of NO. C) Reduced glutathione (GSH) levels in mitochondria isolated from the NO donor-treated cells with or without LA. D) Total NOx level in mitochondrial fraction was markedly elevated after treatment with DETA-NONOate whereas LA supplementation significantly attenuated the elevated amount of NOx. E) Cellular carbonyl levels increased by 71% in mitochondria isolated from NO-treated cells as compared to control cells ($p < 0.05$), whereas addition of LA significantly reduced carbonyl formation. Data from five independent experiments are expressed as the mean \pm SEM.

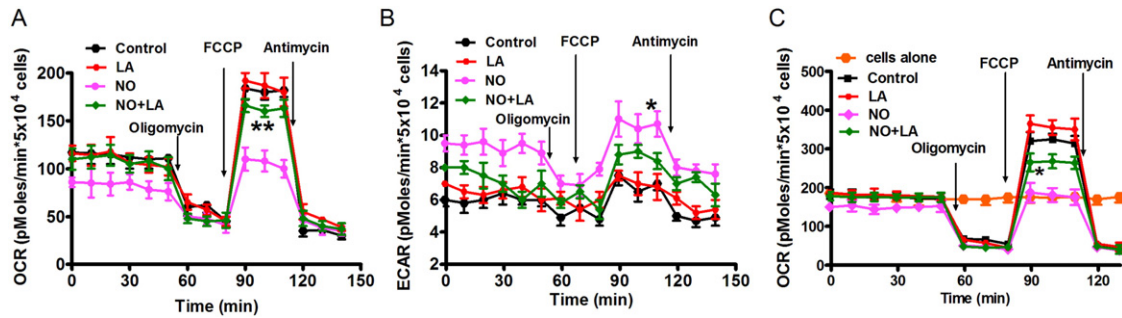


Fig. 3. LA restored OCR and ECAR, inhibited by nitrosative stress in endothelial cells. A) Oxygen consumption rates (OCR), B) Extracellular acidification rate (ECAR) in the primary aortic endothelial cells, and C) OCR in brain endothelial cells, were measured using a Seahorse extracellular flux analyzer. The cells were treated with DETA NONOate (final concentration 250 μ M) for 6 h and then fed LA (50 μ M) for 12 h, followed by the sequential addition of oligomycin, FCCP, and antimycin A as shown. Data are shown as means SEM of $n = 18$ wells (each group) collected from 3 independent experiments. The OCR and ECAR values are normalized by cell numbers.

Mitochondrial protein (25 μ g) was added and the reaction was initiated by the addition of 60 μ M decylubiquinol, and the change in absorbance was monitored for 2 min. To determine antimycin-insensitive activity, the assay was repeated by adding 5 μ g antimycin A to the reaction mixture. Antimycin A-sensitive complex III activity was calculated by comparing the resulting values with the initial rate, which was used as the base value. Enzyme activity was expressed as nmole of reduced cytochrome C per minute per mg of protein.

2.11. Statistical analysis

All data are reported as mean \pm standard error of the mean (SEM). Values of $p < 0.05$ were considered to be statistically significant (95% confidence limits). The significance of the effects of NO, LA, NO + LA and non-treated control groups was compared using one-way ANOVA.

3. Results

3.1. Effect of LA on ATP production, mitochondrial function and redox status

We measured ATP levels in endothelial cells to determine the effect of excess NO on ATP production. We found that after treatment with DETA-NONOate ATP production decreased by 36% compared to control cells and that LA supplementation significantly restored ATP levels (Fig. 2A); it is worth noting that LA alone did not affect ATP levels (Fig. 2A). We measured 4-HNE and GSH concentrations in the mitochondria to evaluate whether or not administration of LA alters oxidative stress and can compensate for possible loss of mitochondrial antioxidant capacity due to RNS. Our results show that the mitochondrial 4-HNE level increased by 57% whereas GSH decreased by ~40% after NO treatment ($p < 0.01$) and that LA-treated cells are less effected by NO. While LA alone did not significantly reduce 4-HNE and increase GSH levels without DETA-NONOate treatment, it suggests that the major

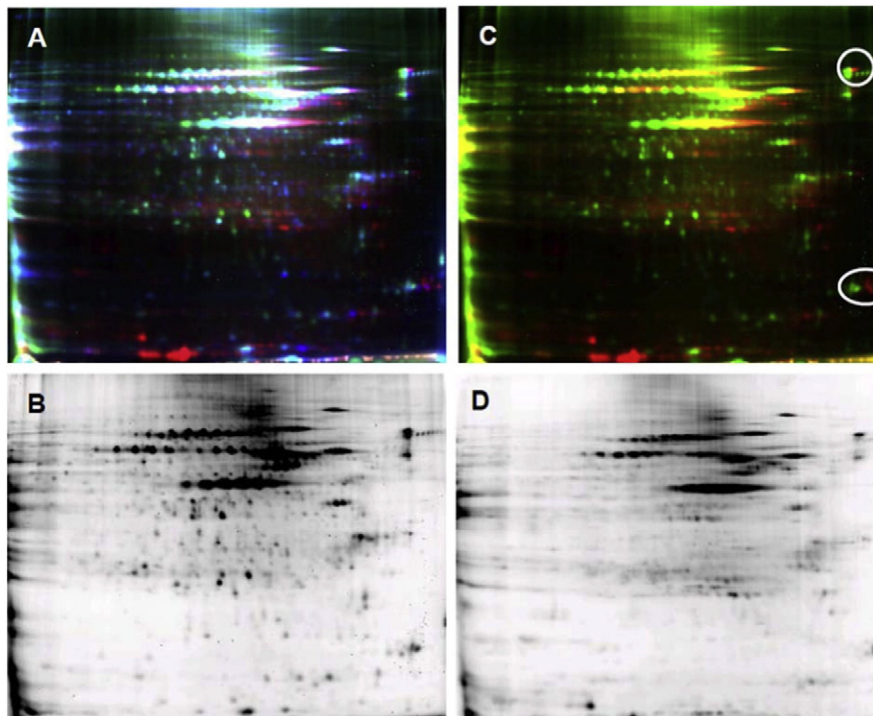


Fig. 4. Overlaid images of SNO proteins. Changes in protein S-nitrosylation as well as changes in total protein expression were analyzed using a modification of the Biotin-switch method. The whole process was illustrated in Fig. 1, and here we show representative images of 2D gels containing S-nitrosylated and non-nitrosylated proteins, and visual evidence that the Cy-maleimide labeling of the modified proteins induces pI shifts. A) NO + LA: Cy2 (blue) is used to labeled LA-treated samples used as control; Cy3 (green) shows proteins treated with NO + LA; Cy5-M (Red) shows nitrosylated proteins. B) Grayscale of Cy3 (Green), i.e., proteins treated with NO + LA. C) NO + LA: Cy3 (Green) is used to label proteins treated with NO + LA; Cy5-M (Red) is nitrosylated proteins. D) Grayscale of Cy5 (red), i.e., Nitrosylated proteins. These images demonstrate 'matching' between total protein and the internal control, while Cy5-M produces a shift in isoelectric point as exemplified by the spots in oval.

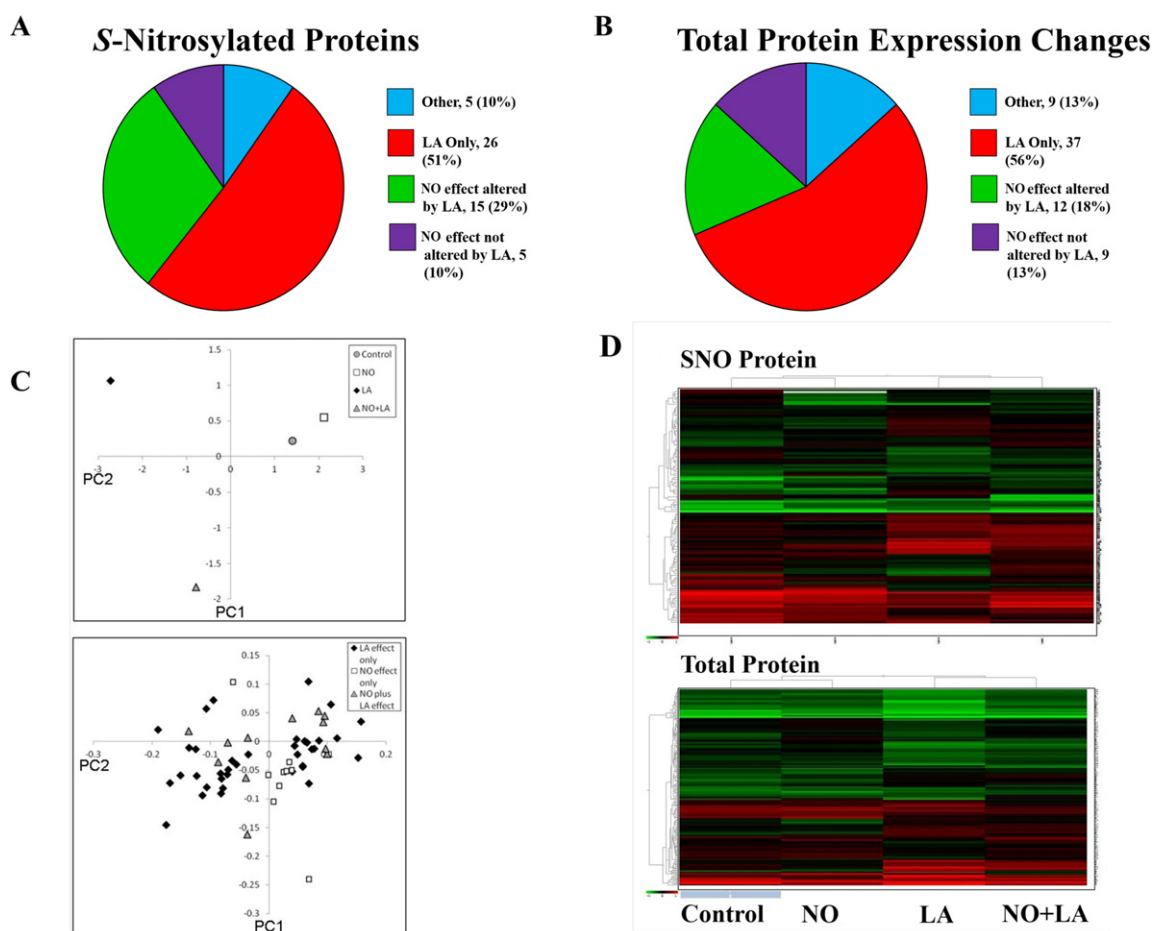


Fig. 5. Total numbers and categories of mass spectrometry-identified A) S-nitrosylated proteins; and B) differentially expressed proteins. C) Principal component analysis (PCA). (Top panel) PCA for SNO proteins. Principal component 1 (PC1) demonstrates that the addition of LA (LA or NO + LA) was the dominant component effecting protein S-nitrosylation. The second principle component (PC2) was between NO + LA and all other treatment groups. (Bottom panel) PCA for total protein expression. The addition of LA (LA or NO + LA) was again the first principal component effecting protein expression. PC2 was between NO + LA and all other treatment groups. D) Hierarchical cluster analysis. With both S-nitrosylated (SNO) and total protein differences LA and NO + LA were clustered together, separate from control and NO which were also clustered together.

effect of LA on 4-HNE and GSH production is mediated through RNS quenching (Fig. 2B and C). In addition, the total NOx level in mitochondrial fraction was markedly elevated after treatment with DETA-NONOate. Supplement with LA significantly attenuated the elevated amount of NO (Fig. 2D), reaching basal levels. Likewise, the ELISA results showed that cellular carbonyl levels increased by 71% in mitochondria isolated from NO-treated cells as compared to control cells ($p < 0.05$), whereas addition of LA significantly reduced carbonyl formation (Fig. 2E). Collectively, these data demonstrate that cells treated with NO have increased levels of RNS and ROS, which are opposed by LA supplementation, partially alleviating the stress.

To determine whether the mitochondrial reserve capacity was altered by excess NO and potentially regulated by LA we examined OCR and ECAR in primary aortic endothelial cells and in brain endothelial cells treated with DETA-NONOate with and without LA supplementation. Mitochondrial reserve capacity was determined by uncoupling oxidative phosphorylation with the proton ionophore FCCP, followed by the addition of mitochondrial respiratory-chain inhibitors. First, oligomycin (5 $\mu\text{g}/\text{mL}$) was added to all samples to inhibit ATP synthase (complex V), and then FCCP (5 μM) was added. Exposure of endothelial cells to FCCP, which uncouples electron flow for ATP synthesis, stimulates respiration to the maximal level and provides an important indicator of mitochondrial reserve capacity (Fig. 3A). Lastly, antimycin A (40 μM) was added to inhibit electron flow through complex III, which causes a dramatic suppression of OCR (Fig. 3A). OCR was significantly decreased in cells exposed to NO (106 ± 11 pmoles/min) compared to OCR basal levels (182 ± 9 pmoles/min), whereas treatment with LA

significantly offset this decline (163 ± 7 pmoles/min; $p < 0.001$). As indicated in Fig. 3B, after the addition of FCCP, NO induced a 50% increase in ECAR compared to control ($p < 0.01$) while LA lowered ECAR by 22.5% ($p < 0.05$). OCR in brain endothelial cells showed comparable values (Fig. 3C), suggesting that the effect of LA on OCR and ECAR is not exclusive to primary-cells.

To determine whether or not LA assists retaining mitochondrial energy production by altering protein S-nitrosylation, we examined changes in total protein levels and S-nitrosylated proteins by a modified 2D DIGE-based biotin-switch method, in which both the traditional 2D DIGE and the biotin-switch approaches were modified. Each 2D gel contained aliquots labeled for S-nitrosylation and total protein from a single sample, and a pooled internal control (Fig. 1). S-nitrosylated

Table 1
S-Nitrosylated proteins that were affected by addition of LA.

Spot no.	Protein identity	Ratio	ANOVA	Matched peptides
771	Heat shock cognate 71 kDa protein	-3.0	0.043	8
880	Isoform C of Prelamin-A/C	-1.25	0.045	6
1097	Protein Prss3	+3.1	0.008	23
1104	Dihydrolipoylysine succinyltransferase of KDC	-2.0	0.037	24
1292	Cytochrome b-c1 complex subunit I	-2.3	0.024	15
1494	β -Actin	-2.0	0.034	16
2031	Prohibitin	-2.2	0.046	12

LA: lipoic acid.

KDC: alpha-ketoglutarate dehydrogenase complex.

Table 2
Proteins that displayed changes in expression affected by LA.

Spot no.	Protein identity	Ratio	ANOVA	Matched peptides
577	Zyxin	−2.1	0.033	8
741	Heat shock 70 kDa protein 5	−2.6	0.025	32
1048	protein disulfide-isomerase	+4.6	0.039	21
1150	Vimentin	−3.2	0.03	36
1319	Ribonuclease inhibitor isoform a	+1.4	0.007	6
1327	Cytochrome b-c1 complex subunit I	+2.0	0.026	15

LA: lipoic acid.

proteins were recognized utilizing Cy5-maleimide (Cy5-M), and changes in expression levels (total protein) were determined by labeling the proteins with NHS-Cy3. Since maleimide-labeled proteins often display shifts in gel migration due to charge changes, an internal protein control, labeled with NHS-Cy2, was used to normalize total protein expression and determine changes in migration patterns. As expected from the Cy5-M labeling, there was an isoelectric point (pI) shift relative to the same sample labeled for total protein with NHS-Cy3 (Fig. 4, A–D). The resulting images for S-nitrosylated proteins and total protein changes were analyzed separately as described previously [29,30].

All the reported results are statistically significant as determined by $p < 0.05$. Differential expression analysis revealed that 51 S-nitrosylated proteins presented expression level changes (Fig. 5A), while total expression levels of 67 of the non S-nitrosylated proteins changed (Fig. 5B). Fifteen (27%) of the 51 differentially S-nitrosylated proteins that were affected by NO were subsequently affected by the LA treatment. Of these, two-thirds of the proteins were characterized as having reduced S-nitrosylation levels resulting from LA treatment (addition of LA to the cell cultures in the presence of NO; data not shown). Addition of LA alone affected 26 (51%) of the S-nitrosylated proteins (Fig. 5A). In contrast, 5 (10%) of the S-nitrosylated proteins that resulted from the effects of NO were not affected by LA supplementation (Fig. 5A). From the 67 non-S-nitrosylated proteins displaying altered protein expression levels, 12 proteins (18%) that were affected by NO were also affected by LA (Fig. 5B); and 37 proteins (56%) were affected by the LA treatment alone (Fig. 5B). Finally, nine of the proteins (13%) that were altered by NO, were not affected by LA.

Statistically significant observations were further corroborated by hierarchical cluster analysis and principal component analysis (PCA). Principal component 1 (PC1) for S-nitrosylated proteins (Fig. 5C, top panel), which contains 55.5% of the variance between samples, confirmed that the addition of LA (LA or NO + LA) is the dominant

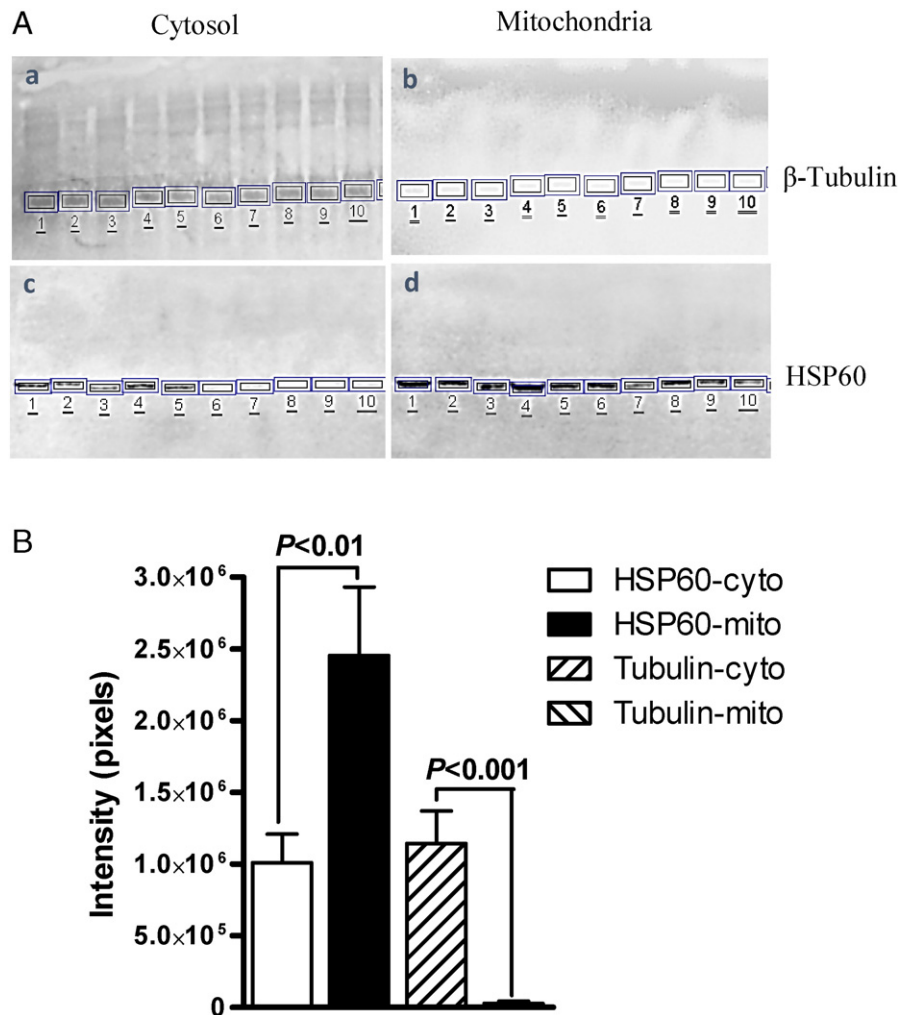


Fig. 6. Activities of complex III and alpha-ketoglutarate dehydrogenase (KDC) in mitochondria isolated from the nitric oxide donor-treated cells with or without LA. A) Purity of mitochondrial preparations assessed by Western blot. Ten randomly selected cytosolic (a and c) and mitochondrial (b and d) fractions were immunoblotted with antibodies against β -Tubulin and HSP60. B) The amounts of the proteins were quantified by Image Studio Light software. Results are given as band intensities of Western blot signals, represented as mean \pm SEM. C) Complex III activity was expressed as nmole of reduced cytochrome C/min per mg of protein. D) KDC activity was determined by monitoring NAD^+ reduction at 340 nm. E and F) Activities of complex III and KDC were increased after adding Cu^+ to the NO-treated group. In the presence of the specific Cu^+ chelator neocuproine (NCP), the effect of Cu^+ was completely abolished. However, adding the Cu^{++} chelator cuprizone (CPZ), had no effect on the enzyme activities. Data from five independent experiments are expressed as the mean \pm SEM.

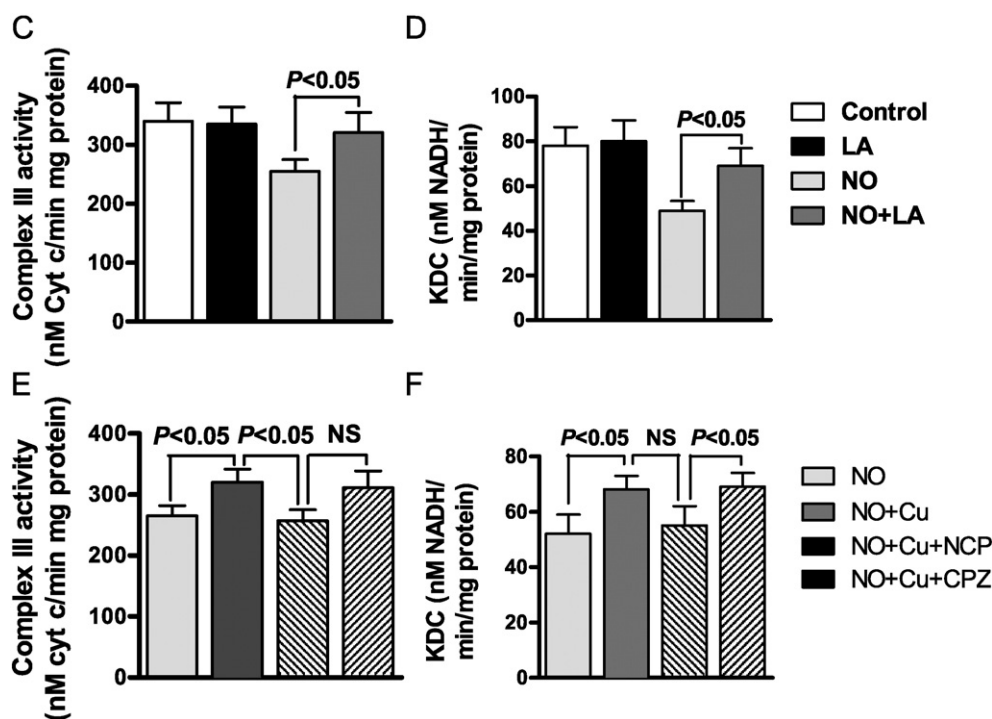


Fig. 6 (continued).

component affecting protein S-nitrosylation. On the other hand, PC2, with 24.2% of variance, is between (NO + LA) and all other treatment groups. For differences in total protein expression, PC1 revealed a similar result with the addition of LA (either LA alone or NO + LA), containing 63.6% of the variance between samples, confirming that LA was also the dominant component effecting protein alteration (Fig. 5C, bottom panel). PC2 of total protein expression, with 21.3% of variance, was also between (NO + LA) and all other treatment groups. Similarly, hierarchical cluster analysis (Fig. 5D) revealed that with both S-nitrosylation and total protein differences, LA and (NO + LA) were clustered together, and were separated from control and NO. Again, this suggests that the addition of LA was the dominant factor regulating protein S-nitrosylation.

Next, seven nitrosylated proteins and six proteins displaying significant changes in expression levels between the NO + LA and the NO groups were identified by MS (Table 1 and Table 2). Among the seven nitrosylated proteins, six proteins treated with (NO + LA) showed decreased S-nitrosylation and one protein showed increased S-nitrosylation levels at least two-fold above that of NO alone. Interestingly, supplementation of NO-treated cells with LA decreased the nitrosylation of subunit I in complex III by 2.3-fold, and increased its expression by 2.0-fold. In addition, LA decreased nitrosylation of dihydrolipoyllysine succinyltransferase by 2.0-fold, a key component of the enzyme KDC.

3.2. Complex III and KDC activities

We found that complex III and KDC, which are primary energy transducing mitochondrial enzymes, are nitrosylated, and that the nitrosylation levels are reduced by adding LA. In order to further evaluate the impact of S-nitrosylation by NO and blocking of nitrosylation by LA on the activities of complex III and KDC, we measured their enzymatic activities in mitochondria isolated from aortic endothelial cells. The purity of mitochondrial fractions was assessed by Western blot analysis and then quantitated by the Image software after normalization mitochondrial protein concentration to cytosol protein concentration. Mitochondrial and cytosolic fractions were immunoblotted with antibodies

against HSP60, a mitochondrial matric protein, and β -tubulin, a cytosolic protein. The results show that distribution of β -tubulin was not able to be visualized in the mitochondrial fractions whereas intensity of HSP60 exhibited a marked increase in mitochondrial fractions (Fig. 6A and 6B). The data clearly indicated a good enrichment of mitochondria following the purification. Complex III and KDC activities in the cells treated with the NO donor dropped 25% and 37%, respectively, whereas LA significantly, restored partially both enzymatic activities ($p < 0.05$) (Fig. 6C and D). To determine whether the alteration of these enzymes' activities could be attributed to S-nitrosylation, we added the mitochondrial isolation buffer (pH 7.4) containing CuCl_2 (10 μM) and ascorbic acid (0.1 mM) to isolated mitochondria, followed by incubation at room temperature for 30 min. It is well known that Cu^{++} is a nitrosylating agent [35,36]. Ascorbic acid was added to reduce Cu^{++} to Cu^+ and prevent Cu^+ spontaneously oxidizing to Cu^{++} . As Fig. 6E and F shows, both enzyme activities were augmented after adding Cu^+ to the NO-treated group. We also found that in the presence of the specific Cu^+ chelator neocuproine (NCP, 100 μM) the effect of Cu^+ was completely abolished. However, adding the Cu^+ chelator cuprizone (bis-cyclohexanone-oxalyldihydrazone, CPZ, 100 μM), had no effect (Fig. 6E and 6F). These data show that the enzyme activities are affected by S-nitrosylation, likely through a Cu^+ -dependent mechanism. By comparing the activity values brought down by adding the NO donor and raised up by adding Cu^+ , we conclude that S-nitrosylation has a major impact in the decline of the enzymatic activities.

4. Discussion

In this study, we have demonstrated that a subunit of complex III in the electron transport chain, and dihydrolipoyllysine succinyltransferase, a subunit of KDC in the Krebs cycle, are susceptible to S-nitrosylation in response to NO treatment. In addition, S-nitrosylation of complex III and KDC coincide with a significant decrease in the activities of these two enzymes. These data demonstrate that S-nitrosylation decreases enzyme activities. Although canonical NO signaling occurs likely through its interaction with soluble guanylyl cyclase and controls cyclic guanosine monophosphate (cGMP)-dependent signaling, some investigations

have suggested that protein S-nitrosylation is an essential component of vascular (dys)function [6]. The endothelium is an ideal site for S-nitrosylation-dependent signals because endothelial cells constitutively express the NO-producing enzyme endothelial nitric oxide synthase (eNOS), and under inflammatory conditions, it is also exposed to nitric oxide-derived from the inducible NOS isoform (iNOS) [14]. Our findings support these observations. Importantly, our results reveal that LA supplementation significantly reduces S-nitrosylation on KDC and complex III, and partially restores their activities as well. These results disclose that regulation of protein S-nitrosylation is a novel redox regulatory mechanism of LA by which LA quenches NS to retain normal energy homeostasis. Our results agree with observations by other investigators. For instance, Murphy et al. found that KDC could be nitrosylated and that the nitrosylated form of the enzyme becomes inactive [9]. On the other hand, the fact that LA cannot completely abolish elevated levels of NO and fully restore the enzyme activity suggest that besides protein S-nitrosylation, other forms of regulations may exist. For example, cGMP dependent signal pathway and peroxynitrite could be part of these alternative mechanisms. It is well established that some biological activities including regulation of mitochondrial biogenesis [37] by NO are mediated via its primary receptor, the soluble guanylyl cyclase (sGC) [38]. In addition, NO reacts readily with superoxide to produce peroxynitrite, which can inhibit mitochondrial functions. Furthermore, our data show that the impairment of OCAR/ECAR and the mitochondrial reserve capacity after Deta-NONOate administration were readily reversible upon the addition of LA. These observations suggest that this rapid reversible reaction is not likely due to protein S-nitrosylation, since covalent modifications are not usually reversible. Since free NO can be converted into SNO and SNO can convert to NO, our current data do not support any effect over the other; i.e., which effect is mediated through free NO released from the NO donor or through S-nitrosylation modification.

We propose that diminished activities of complex III and KDC may lead to the decreased ATP production observed in our experiments for the following reasons: 1) complex III is a critical component of all primary energy transduction systems. It functions in maintaining the proton gradient, which provides ~30% of body energy [39]. 2) KDC is a critical regulatory enzyme in the Krebs cycle [40], limiting the NADH production rate and oxidative phosphorylation [41]. It is thought that KDC plays a key role in bioenergetic deficit associated with oxidative stress [42]. 3) It is well-established that NO interacts with the mitochondrial respiratory chain by inhibiting cytochrome c oxidase (complex IV) and NADH-ubiquinone oxidoreductase (complex I) [43,44]. And 4) mitochondrial proteins are constantly exposed to excess ROS [45]; therefore, are susceptible to oxidative damage, leading to energy deficiency [46]. Inhibition of mitochondrial complexes in the electron transport chain by excess NO leads to enhanced production of ROS [47]. Our data revealed that levels of 4-HNE—a biomarker of lipid peroxidation [48], carbonylated proteins—a biomarker of protein oxidation [49], and nitric oxide, were significantly increased whereas GSH levels were significantly decreased in the mitochondrial fractions. 4-HNE is derived from peroxidation of n-6 polyunsaturated fatty acids such as arachidonic and linoleic acids and it is one of the most abundant and active lipid peroxides. 4-HNE reacts with amino acids, such as cysteine, lysine or histidine, and forms stable adducts with proteins, leading to modulating activities of proteins. Carbonyl groups (aldehydes and ketones) are produced on protein side chains (especially of Pro, Arg, Lys, and Thr) where they are oxidized and increased. These alterations are partially restored by LA supplementation. The data suggest that LA may provide mitochondrial protection against NO damage, likely through regulation of GSH, a predominantly intra-mitochondrial antioxidant that protects mitochondrial complexes from NO-induced damage [50].

Our proteomics experiments indicate that most of the nitrosylated proteins affected by LA supplementation are involved in protein folding like protein disulfide-isomerase (PDI) and heat shock cognate 71 kDa

(Hsc70). LA treatment affected the nitrosylation levels of PDI and Hsc70 by 4.6-fold increase and 3.0-fold decrease, respectively. PDI contains two thioredoxin domains that catalyze the formation, breakage, and rearrangement of disulfide bonds [51]. Hsc70 is involved in chaperoning unfolded proteins for degradation by both the ubiquitin-proteasome system and liposomes [52]. We do not know whether these observations could provide more evidences to support McCarty's hypothesis. He postulated that LA could induce a heat shock response by assisting the formation of intra-molecular disulfide bonds in signaling proteins [53].

We have shown here that the expression of structural proteins including zyxin, prohibitin, vimentin, lamin A/C and actin is affected by excess NO, and changed by LA supplementation. We also observed that in response to excess NO, nitrosylation of actin and vimentin increased and appeared to decrease by LA supplementation. Zyxin is a protein attached to the cytoskeleton helping to organize function, dynamics and maintenance of actin fibers [54]. In endothelial cells, zyxin is activated in response to stretch, followed by translocation from the cytoskeleton to the nucleus [55]. Prohibitin (PHB) acts as a mediator of mitochondria shuttling in response to oxidative stress [56]. PHB also acts as a molecular chaperone stabilizing newly synthesized respiratory enzymes in mitochondria [57]. It seems clear that LA exerts multiple regulatory effects beyond direct ROS quenching. These regulatory functions may involve altering protein S-nitrosylation and regulation of the expression levels of some proteins that may be affected by S-nitrosative mechanisms. Our findings also show that mitochondrial proteins, regardless of their cytosolic or mitochondrial origin, and cytosolic proteins such as zyxin and Hsc70, undergo S-nitrosylation in the presence of elevated NO.

In summary, LA supplementation effectively restores mitochondrial enzyme activities of complex III and the α -ketoglutarate dehydrogenase complex inhibited by excess NO, mainly via S-nitrosylation. Regulation of these two enzymes, particularly via reducing S-nitrosylation contributes to the protective effect observed. Our studies shed light on a new mechanism of antioxidant activity of LA, and suggest a strategy for the treatment of diseases in which chronic inflammation is involved.

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