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Skeletal Muscle Aldolase an Overexpression in Endotoxemic Rats and Inhibited by GSNO *via* Potential Role for *S*-nitrosylation *In Vitro*

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Background. **Hepatic aldolase (ALD) A mRNA transcription and ALD B *S*-nitrosylation have been confirmed in endotoxemic rats and mice, respectively. In the present study we investigated whether the skeletal muscle ALD A shared potential for *S*-nitrosylation to act as a hypoxiarelated signaling mechanism in lipopolysaccharide (LPS) challenged rats.**

Materials and methods. **Male Sprague Dawley rats were treated (i.p.) as follows, control group ($n = 6$) with 0.9% NaCl, tested group ($n = 6$) with a single dose of 2 mg/kg LPS. Protein *S*-nitrosylation was determined by biotin switch and dot blotting analysis. ALD A, hypoxia-inducible factor 1α and vascular endothelial growth factor were determined by western blotting. ALD A catalytic activity treated with *S*-nitrosoglutathione (GSNO), an exogenous NO-donor, was examined *in vitro*.**

Results. **There were several *S*-nitrosylated proteins under basal conditions. ALD A was over-expressed in a hypoxia-related way in the skeletal muscle of LPS challenged rats. Importantly, treatment of ALD A with GSNO at concentration 50 μmol/L ~ 1000 μmol/L that inhibited catalytic activity, increased the number of *S*-nitrosylated bands and led to hyper-nitrosylation of basally *S*-nitrosylated proteins of ALD A. Quantization of enzyme *S*-nitrosothiol showed that a maximal of four cysteines per subunit was modified by *S*-nitrosylation in the presence of GSNO.**

Conclusions. **These findings suggested that *S*-nitrosylation of ALD A might serve as a novel mechanism for controlling ALD A activity at the post-translational level in endotoxemic rats.**

Key Words: **aldolase A; *S*-nitrosylation; *S*-nitrosoglutathione; hypoxia related signaling mechanism; endotoxemic rats.**

INTRODUCTION

Aldolases (ALDs) are glycolytic enzymes catalyzing the reversible cleavage of fructose-1, 6-bisphosphate (Fru-1, 6-P2) into glyceraldehyde-3-phosphate (G3P) and dihydroxyacetone phosphate (DHAP) [1]. Encoded by three different genes, ALD A binds to the actin-containing filament of the cytoskeleton. ALD B is expressed in the liver cytoskeleton, and ALD C is expressed in brain and other nervous tissue. ALDs may exert a lot of functions besides those characteristics of glycolytic enzymes. They have a close relationship with the muscle damage, the development of brain, and ATP production [2]. To date, it has been found that ALD isoenzyme participates in energy metabolism, histogenesis and so on, however, little is known about the roles for its modification and related regulation mechanisms.

Protein *S*-nitrosylation, the covalent attachment of a NO group to the cysteine(s) of specific proteins, is recognized as a novel post-translational modification regulating a diversity array of protein functions [3, 4]. However, due to technical difficulties, only a very limited number of proteins susceptible to *S*-nitrosylation have been identified [5]. By using matrix-assisted laser dissociation/ionization time-of-flight mass spectrometry (MALDI-TOF MS) and adopting biotin method, we identified several proteins that are susceptible to *S*-nitrosylation in liver microsomes, including ALD B,

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which might be *S*-nitrosylated *in vivo* after lipopolysaccharide (LPS) challenge [6]. However, not to mention the uncertainty of functional consequences, the fact that biotin switch method is now being extensively criticized as nonspecific in identifying individual *S*-nitrosylated proteins [7] highlights the necessity to examine in details whether ALD A is indeed *S*-nitrosylable and, thereby, regulated.

Chemical modification studies have shown that cysteine modifications often bring an alteration in enzymatic activities [8–10]. *S*-glutathionylation, the formation of a mixed disulfide between protein cysteine(s) and a low molecular thiol glutathione, was further demonstrated to modulate ALD A activity [11, 12]. These data implied that cysteine modification by *S*-nitrosylation might also play a role in controlling ALD A activity.

The present study was carried out to examine the expression of ALD A in skeletal muscle of LPS challenged rats and/or treated with a physiologically existing NO donor, *S*-nitrosoglutathione (GSNO) *in vitro*. Furthermore, whether ALD A was *S*-nitrosylable after NO donor treatment was determined. The catalytic alteration of ALD A was also examined in parallel. Due to the fact that they are important molecular events in hypoxia related signaling pathway, hypoxia-inducible factor 1α (HIF-1α) and vascular endothelial growth factor (VEGF) were hypothesized to be involved in the expression and activity of ALD A in hypoxia condition. We investigated whether the skeletal muscle ALD A shared potential for *S*-nitrosylation to act as a hypoxia related signaling mechanism in LPS challenged rats.

MATERIALS AND METHODS

Materials

Bacterial LPS (derived from *Salmonella abortus equi*) was purchased from Sigma-Aldrich Biotechnology (St. Louis, MO, USA). Rabbit skeletal muscle ALD A, streptavidin agarose, methyl methanethiosulfonate (MMTS), neocuproine, and dimethylformamide (DMF) were obtained from Sigma. Monoclonal antibody to nitrosocysteine was from A.G. Scientific (San Diego, CA, USA). Saphead G-25 (fine) and enhanced chemiluminescence reagents were purchased from Pharmacia (Basking Ridge, NJ, USA). ALD A Activity Test Kits was purchased from Nanjing Jiancheng Technology (Nanjing, China). The primary ALD antibody was purchased from ABCam Biotechnology (Cambridge, UK) and the primary HIF-1α and VEGF antibody from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Horseradish peroxidase (HRP)-conjugated goat anti-rabbit and rabbit anti-mouse antibody were purchased form MultiSciences Biotechnology (Hangzhou, China). Peroxidase-conjugated goat anti-mouse IgG were from Santa Cruz Biotechnology, and 2,3-diaminonaphtylene was from Acros (Geel, Belgium). Enhanced chemiluminescent substrate (ECL) and *N*-[6-(biotinamido) hexyl]-3′-(2′-pyridyldithio) pro-pionamide (biotin-HPDP) was purchased from Pierce (Rockford , IL, USA).

Synthesis of GSNO

GSNO was synthesized by combining equimolar (200 mmol/L) $NaNO_2$ and glutathione in 0.5 mol/L HCl containing 50 μmol/L diethylene triamine pentaacetic acid (DTPA) in the dark at room temperature for 10 min as described previously [13] . Before use, the GSNO solution was neutralized to pH 7.0 with 0.1 mol/L NaOH. This solution was prepared daily to avoid storage derived decomposition.

Preparation of Skeletal Muscles in Endotoxemic Rats

Male Sprague-Dawley rats (weighing 180–250 g) were housed individually and maintained with standard rat chow and tap water on a 12-h light/12-h dark cycle. The experiment conformed to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health, and approval was granted by the ethics review board of Zhejiang University. For *in vivo* experiments, 12 rats divided into two groups ($n = 6$ rats/group) were given a single dose injection (i.p.) of NaCl or LPS (2 mg/kg). After 6 h, rats were sacrificed and the skeletal muscles were prepared and kept on ice.

Western Blotting

Rat skeletal muscle tissues were lysed with RIPA buffer for 30 min on ice. Samples were clarified by centrifugation for 30 min at 13000 $\times$ *g* at 4°C. Each sample was separated by SDS-polyacrylamide gel, then transferred onto PVDF membranes for 90 min and blocked for 1 h, followed by an overnight incubation at 4°C with respective antibody (anti-ALD A at 1:4000 dilution, anti-VEGF at 1:500 dilution, anti-HIF-1α at 1:400 dilution, anti-GAPDH at 1:10,000 dilution). Then the membranes were incubated with horseradish peroxidase HRP-conjugated goat anti-rabbit, rabbit anti-goat, or mouse anti-mouse antibody at 1:5000 dilution. The proteins were visualized antoradiographically with ECL, and scanned using a bioimaging analyzer (Bio-Rad, Hercules, CA, USA). The density of the products was quantitated using Quantity One ver. 4.2.2 software (Bio-Rad).

Treatment with GSNO

The rat skeletal muscles were homogenated by Tris-HCl buffer on ice. Samples were clarified by centrifugation for 30 min at 13,000 $\times$ *g* at 4°C. Sample protein final concentration was 0.5 mg/mL, with quantification by Lowry analysis. Fresh GSNO of varying concentrations of 50, 200, 500, and 1000 μmol/L were subsequently added to the samples, and incubated for 1 h at room temperature.

Pure ALD A (0.1 mg/mL) was incubated with freshly prepared GSNO at final concentrations of 50, 200, and 1000 μmol/L in the dark for 30 min. Samples were then diluted 200-fold to measure the catalytic activity. For quantization of enzyme *S*-nitrosothiols, samples were passed a Sephadex G25 (fine) column to eliminate the excessive GSNO.

Measurement of ALD A Activity

ALD A activity was determined by ALD kit. In brief, the rate of Fru-l, 6-P2 cleavage was measured according to ALD A kit instruction. Samples (20 μL) from rat skeletal muscles were detected with the substrate Fru-1, 6-P2 concentration of 0.062, 0.185, 0.556, 0.1667, and 5.000 mmol/L following the Test Kits description.

Pure ALD A (5 μg/mL) treated with or without GSNO was added to a solution containing 50 mmol/L Tris-HCl buffer, pH 7.5, 5 mmol/L EDTA, 2.5 mmol/L Fru-l, 6-P 2, 0.2 mmol/L NADH, and 10–15 μL of a stock solution of a-glycerophosphate dehydrogenase and triose phosphate isomerase.

One unit of ALD A activity was defined as the cleavage of 1 μmol/min of Fru-l, 6-P2. ALD A concentration was determined spectrophotometrically at 280 nm, assuming a molecular weight of 160 kDa [1]. Km and Vmax were analyzed by GraphPad Prism 4 software (Graphpad Software Inc, San Diego, CA, USA).