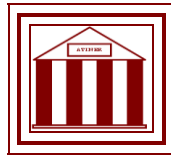# Athens Institute for Education and Research
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PHA2016-2028

Oxidative Stress in Skeletal Muscle:
The Role of Nrf2 and Effect of α-lipoic Acid

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Oxidative Stress in Skeletal Muscle: The Role of Nrf2 and Effect of α-lipoic Acid

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Abstract

Lipoic acid (LA) is a natural antioxidant which alleviates oxidative stress parameters in skeletal muscle. Nrf2 is a regulator of cellular resistance to oxidants, whose activation can protect muscle against the oxidative stress induced by lipopolysaccharide (LPS). The aim of the study was to determine whether the antioxidant effect of lipoic acid during LPS-induced oxidative stress is mediated through the Nrf2 pathway. Experiments carried out on rats, treated intraperitoneally with saline or LA (60 mg/kg) 30 minutes after LPS administration. After five hours of observation, the animals were euthanized. Their femoral muscles were isolated for the measurements of lipid peroxidation, Nrf2 expression and the concentration of hydrogen peroxide (H_2O_2), and free sulfhydryl groups (-SH). The injection of LPS alone resulted in the development of shock and oxidative stress, indicated by a significant increase in skeletal muscle lipid peroxidation and H_2O_2 concentration and a decrease in the content of free-SH groups in skeletal muscle. Administration of LA following the LPS challenge resulted in higher total -SH group concentration, greater Nrf2 expression, and lower lipid peroxidation and H_2O_2 concentration in the skeletal muscle compared with the LPS groups. The results indicate that LA treatment effectively protected the skeletal muscles against endotoxin-induced oxidative stress, and that this effect is mediated through Nrf2 pathways.

Keywords: Lipoic acid, Nrf2, Oxidative stress.

Introduction

Skeletal muscles contain different types of muscular fibre, with a range of structural properties and functional capabilities. Lipopolysaccharide (LPS) is considered a powerful inducer of skeletal muscle atrophy in mammals resulting in the activation of catabolic signaling pathways which feed forward to the nucleus to promote the activation of muscle atrophy. Skeletal muscle atrophy during sepsis is characterized by progressive loss of strength and muscle mass, increased MHC (myosin heavy chain) degradation, and an increase in the expression of atrogin-1 and MuRF-1 (muscle-specific RING-finger protein 1). Also ubiquitin-protein ligases (E3S) belonging to the ubiquitin-proteasome system are critical in the development of muscle atrophy (Hasselgren et al., 1986; Hasselgren et al., 1989). Multiple investigations have shown that muscle atrophy in sepsis mainly results in increased protein breakdown (Bodine et al., 2001; Gomes et al., 2001) via the ubiquitin proteasome pathway (Tiao et al., 1994; 1997).

LPS, a bacterial endotoxin from Gram-negative bacteria, is a trigger of the systemic inflammatory response in sepsis. LPS is a potent activator of toll-like receptors (TLRs) contributing to the production of powerful inflammatory mediators including tumor necrosis factor (TNF-α), interleukin-1 (IL-1), IL-1β and IL-6, which are believed to be the direct cause of LPS toxicity (Beutler, 2000; Takeda et al., 2003; Jeger et al., 2015). Activation of TLR4 by LPS causes the dissociation of regulatory NF-kB subunits p50 and p65 from inhibitor NF-kB (I-κB), their translocation to the nucleus increased transcription of genes coding for cytokines (i.e.: TNF-α) and subsequent inflammation (Gosh and Hayden, 2008; Chen and Greene, 2004; Gilmore, 2006).

Oxidative stress is characterized as an imbalance between the production of oxidants, such as hydrogen peroxide (H_2O_2) and superoxide anions ($O_2 \cdot -$), and the efficiency of antioxidant defence mechanisms, resulting in cellular apoptosis or necrosis. ROS damage many cellular components including DNA, lipid membranes and proteins (Aoi et al., 2004; Kozlovsky et al., 1997). However, cells also possess a variety of antioxidant defence systems, including such specific antioxidant enzymes as superoxide dismutase (SOD), catalase (CAT), heme oxygenase (HO) and glutathione peroxidase, which either convert oxygen radicals into less aggressive products or prevent their formation (Devasagayam et al., 2004; Kozlovsky et al., 1997).

Other antioxidants include small molecules such as ascorbic acid and lipoic acid, which may intervene as sacrificial molecules in redox cycles (Harris, 1992).

Lipoic acid (1,2-dithiolane-3-pentanoic acid) is a disulphide compound and a natural coenzyme of pyruvate dehydrogenase and α-ketoglutarate dehydrogenase. It is synthesized enzymatically in the mitochondrion from octanoid acid and may also be derived from exogenous dietary sources (Shay et al., 2009). Lipoic acid and its reduced form, dihydrolipoic acid, are present in all kinds of eukaryotic cells, which have capacity to (i) scavenge reactive species, (ii) regenerate endogenous antioxidants such as

glutathione, acetyl-L-carnitine and vitamin C and E, and (iii) chelate metals (Arroll et al., 2014; Packer et al., 1995; Shen et al., 2008).

The key role in oxidative stress is played by the transcriptional nuclear factor erythroid-2-related factor 2 (Nrf2), which activates detoxifying enzymes by binding to antioxidant response elements (AREs) in target genes (Lee et al., 2005; Kensler et al.; 2007). The transcription of these genes enhances cellular resistance against oxidative stress. Lipoic acid was shown to activate Nrf2 in various animal models including myocardial ischemia/reperfusion (Deng et al., 2013), arsenic exposure (Huerta-Olvera at al., 2010; Lau et al., 2013) and acetaminophen intoxication (Elshazly et al., 2014; Wagner et al., 2012), indicated that treatment myotubes LA and Q10 caused an increase in glutathione, which was associated with increased levels of nuclear Nrf2 protein (Zhao et al., 2015) indicated that LA and lipoamide stimulated expression of Nrf2 in retinal pigment epithelial cells.

The aim of the study determined whether the antioxidant effect of LA observed during oxidative stress induced by LPS is mediated through the Nrf2 pathway.

Materials and Methods

Animals

All experiments were carried out on male Wistar rats aged 2-3 months. The rats were found to weigh 200-230 g directly before testing. The animals were kept under standard laboratory conditions, i. a. temperature of $20 \pm 2°C$, and lighting from 6:00 to 18:00, with free access to lab chow and tap water, until used in the study. All animals were maintained for one week in the laboratory for adaptation. The experimental procedures followed the guidelines for the care and use of laboratory animals, and were approved by the Medical University of Lodz, Ethics Committee No. 20/Ł418/2008.

Experimental Protocol

The rats were randomly assigned to four experimental groups as follows:

Group I (control, n=8) received two 0.2 ml doses of saline with half an hour between them;

Group II (LPS, n=8) received 0.2 ml of saline, and half an hour later, endotoxic shock was induced by injection of *Escherichia coli* lipopolysaccharide (LPS) at a dose of 15 mg/kg;

Group III (LA, n=8) received 0.2 ml of saline, and half an hour later, the rats were injected with a single dose of lipoic acid (60 mg/kg);

Group IV (LPS/LA, n=8) received a single dose of LPS (15 mg/kg) followed by a single dose of LA (60 mg/kg) half an hour later.

All agents were injected intravenously into the tail vein between 8.00 and 9.00 a.m.

Tissue Preparation and Collection of Samples

Five hours after the final injection, the rats were sacrificed. Skeletal muscle was excised from the right thigh and rinsed with ice-cold saline to remove excess blood. The muscle was dried by blotting between two pieces of filter paper, weighed and frozen at -75°C until used for measurements. All steps were carried out at 0-4°C.

Determination of Lipid Peroxidation

The lipid peroxidation product content in skeletal muscle homogenates was assayed as TBARS, previously described by Yagi (Yagi et al., 1986). Briefly, 50 mg of frozen tissue was homogenized in 2 ml of 1.15% KCl using a glass homogenizer. Next, 1 ml of homogenate was boiled for 15 minutes in a mixture containing 0.015% butylated hydroxytoluene, 0.375% thiobarbituric acid and 15% trichloroacetic acid. After cooling, 2.5 ml of butanol was added, vortexed and centrifuged at 3,000 rpm for 10 minutes. Thiobarbituric acid reactive substances (TBARS) were measured spectrofluorometrically using an LS-50 Perkin Elmer Luminescence Spectrometer (Norwalk, CT, USA). Excitation was set at 515 nm and emission was measured at 546 nm. Readings were converted into μM using the calibration curve obtained for 1,1,3,3-tetramethoxypropane (0.01–50 μM). Finally, the results were calculated for 50 mg of lung tissue.

Determination of H_2O_2

Generation of H_2O_2 in skeletal muscle homogenates was determined according to Ruch et al. (Ruch et al., 1983). Samples of 50 mg of frozen skeletal muscle tissue fragments were homogenized with 2 ml of 1.15% KCl, and a 10 μl aliquot of the resulting homogenate was divided between two Eppendorf tubes. In one tube, a mixture of phosphate buffered saline (PBS) (pH 7.0) and horseradish peroxidase (HRP) (1 U/ml) containing 400 μmol homovanillic acid (HVA) was added to assay HRP + HVA, while PBS and 1 U/ml HRP were added to the other tube to assay HRP. Both tubes were simultaneously incubated for 60 minutes at 37°C. Subsequently, PBS and 0.1 M glicyne-NaOH buffer (pH 12.0) with 25 mM EDTA were added to each Eppendorf tube to stop the enzymatic reaction. Excitation was set at 312 nm and emission was measured at 420 nm (Perkin Elmer Luminescence Spectrometer, Beaconsfield, UK). Readings were converted into H_2O_2 concentration using the regression equation prepared from three series of calibration experiments with 10 increasing H_2O_2 concentrations (range 10–1000 μM).

Determination of the Total -SH Group Content in Skeletal Muscle Homogenates

The -SH group content in the skeletal muscle homogenates was determined by the 2.2-dithiobisnitrobenzoic acid (DTNB) assay described by Ellman (Ellman, 1970).

RNA Isolation

Total RNA was extracted from samples using RNeasy mini kits (Qiagen). Briefly, frozen samples of rat skeletal muscle were homogenized in 300 µL of RLT Buffer by Tissue Ruptor homogenizer (Qiagen). Following this, 590 µL of Nuclease-Free Water (Ambion) and 10 µL of Qiagen Proteinase K solution were added. The homogenates were incubated at 55°C for 10 minutes and centrifuged for three minutes at 14,000 rpm. The following part of the protocol was performed as described by the manufacturer. RNA was quantified using a Pico Drop spectrophotometer (Picodrop Limited, UK). The quality of RNA samples was analyzed by measuring the ratio of absorptions at 260/280nm.The purified total RNA was immediately used for cDNA synthesis or stored at -80°C. cDNA generation was performed with a Quanti Tect Reverse Transcription Kit (Qiagen) according to the protocol of the manufacturer, with 1 µg of total RNA used as a starting material. Reverse transcription was performed in conditions optimized for use with this kit (25°C for 10 minutes, 37°C for 120 minutes, and 85^{0}C for 5 minutes). The cDNA samples were kept frozen at -20°C.

Determination of Nrf2 mRNA Expressions

Real Time PCR Analysis. mRNA quantification was performed using standard TaqMan Gene Expression Assays (Applied Biosystems), with Nfe2l2 (Assay ID: Rn00477784 m1) and Actb (Rn00667869 m1) as a control. The 20 µL qPCR included 50 ng cDNA, 10µL TaqMan Universal PCR Master Mix, and 1 µL TaqMan Gene Expression Assay (20x). The reactions were incubated in a 96-well plate at 95°C for 10 minutes, followed by 40 cycles of 95°C for 15 s and 60°C for one minute. All reactions were run in triplicate. TaqMan PCR assays were performed on a 7900HT Fast Real-Time PCR System (Applied Biosystems) and analyzed using Sequence Detection System 2.3 Software. Fold induction values (RQ) were calculated according to the equation 2−ΔΔCt, where ΔCt represents the differences in cycle threshold numbers between the target gene (Nrf2) and endogenous control (β-actin) and ΔΔCt represents the relative change in these differences between the examined and control groups.

Statistical Analysis

STATISTICA 12 (StatSoft) software was used to perform statistical calculations. The results were presented as means ± SEM. The differences

between two groups were evaluated using the independent Student's *t*-test. Values of $p < 0.05$ were accepted as statistically significant.

Results

Effect of lipoic acid on oxidative stress parameters.

Content of TBARS in Skeletal Muscle

Intravenous administration of a single dose of LPS (15 mg/kg b.w.) induced severe oxidative damage to the skeletal muscle. The muscle skeletal tissue of LPS-treated rats showed increase in the TBARS concentration when compared to control rats ($p < 0.001$). Moreover, this increase was significantly attenuated by the treatment with LA (60 mg/kg b.w.) after endotoxin administration ($p < 0.05$) (Figure 1).

Figure 1. *Effect of Lipoic Acid (LA) (60 mg/kg), Lipopolysaccharide (LPS) (15 mg/kg) and Their Combination on TBARS Concentration in Skeletal Muscle Homogenates. Values are Expressed as Means ± SEM (n = 8 rats)*
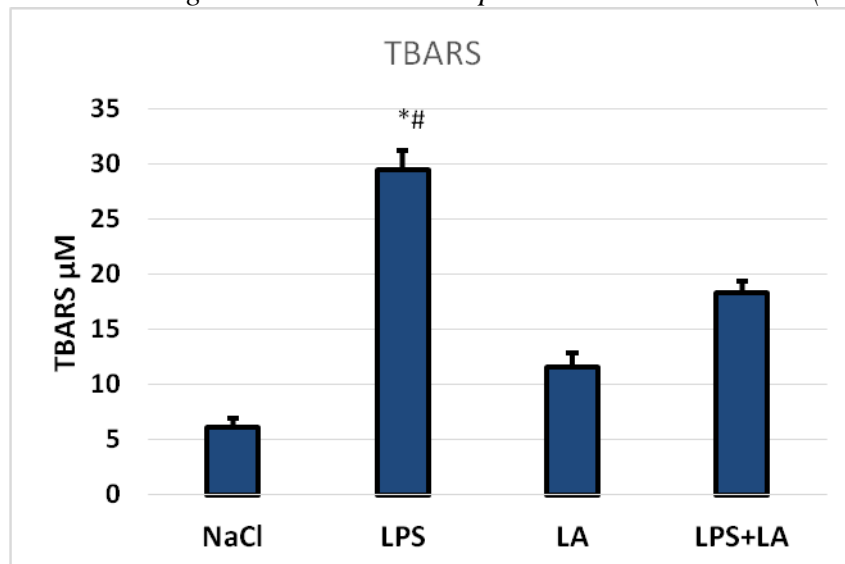

$p<0.001$ vs NaCl and LA, #$p<0.05$ vs LPS+LA

Content of H_2O_2 in Skeletal Muscle

The skeletal muscle tissue of LPS treated rats demonstrated a significant increase ($p < 0.001$) in the levels of H_2O_2 when compared to control rats. Tissue from rats receiving LA supplementation (60 mg/kg b.w) after LPS administration showed a remarkable decrease ($p < 0.001$) in H_2O_2 levels, indicating mitigation of oxidative damage to the skeletal muscle tissue (Figure 2).

Figure 2. *Effect of Lipoic Acid (LA) (60 mg/kg), Lipopolysaccharide (LPS) (15 mg/kg) and Their Combination on H_2O_2 Levels in Skeletal Muscle Homogenates. Values are Expressed as Means ± SEM (n = 8 rats)*
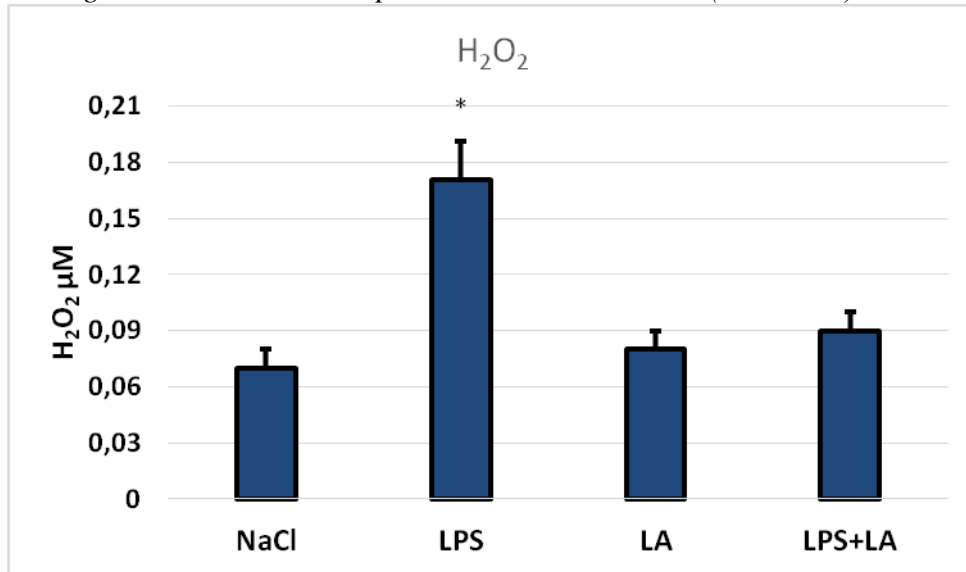

p < 0.001 vs NaCl, LA and LPS+LA

Content of Total -SH Groups in the Skeletal Muscle

Rats treated with LPS had significantly ($p < 0.002$) lower levels of -SH groups than rats treated with LA. Administration of LA (60 mg/kg b.w.) after LPS challenge significantly increased the content of -SH groups in the skeletal muscle homogenates when compared to the LPS group. ($p < 0.001$) (Figure 3).

Figure 3. *Effect of Lipoic Acid (LA) (60 mg/kg), Lipopolysaccharide (LPS) (15 mg/kg) and their Combination on -SH Groups Concentration in Skeletal Muscle Homogenates. Values are Expressed as Means ± SEM (n = 8 rats)*
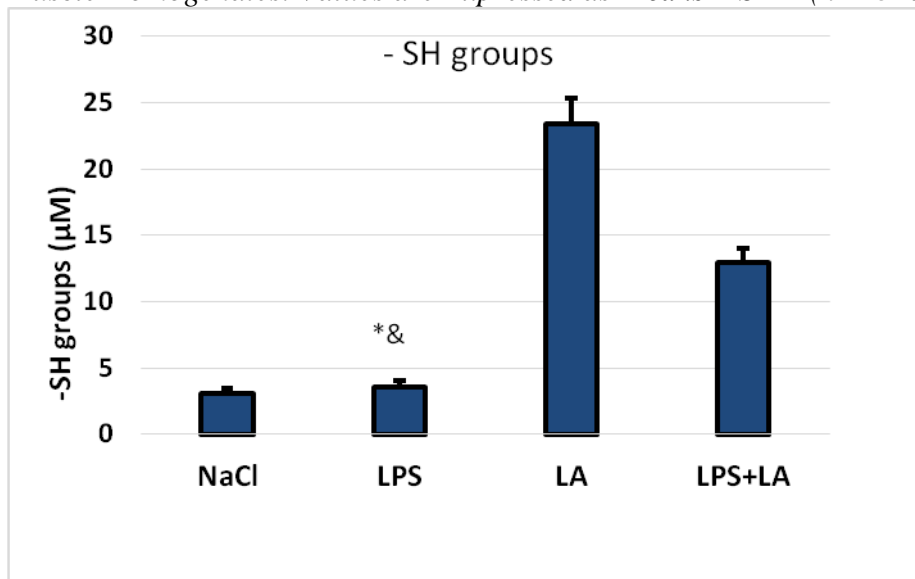

p<0.002 vs LA, &p<0.001 vs LPS+LA

LA Administration Alters the Expression of Nrf2 during Endotoxemia

Figure 4 presents Nrf2 mRNA expression levels in the rat skeletal muscles. Nrf2 mRNA expression is significantly decreased in the LPS group when compared to the LA group ($p < 0.05$). Administration of LA alone successfully activated the expression of Nrf2 compared to the LPS+LA group ($p < 0.05$).

Figure 4. *Effect of Lipoic Acid (LA) (60 mg/kg), Lipopolysaccharide (LPS) (15 mg/kg) and Their Combination on Nrf2 mRNA Expression Levels in Skeletal Muscle Homogenates. Values are Expressed as Means ± SEM (n = 8 rats)*
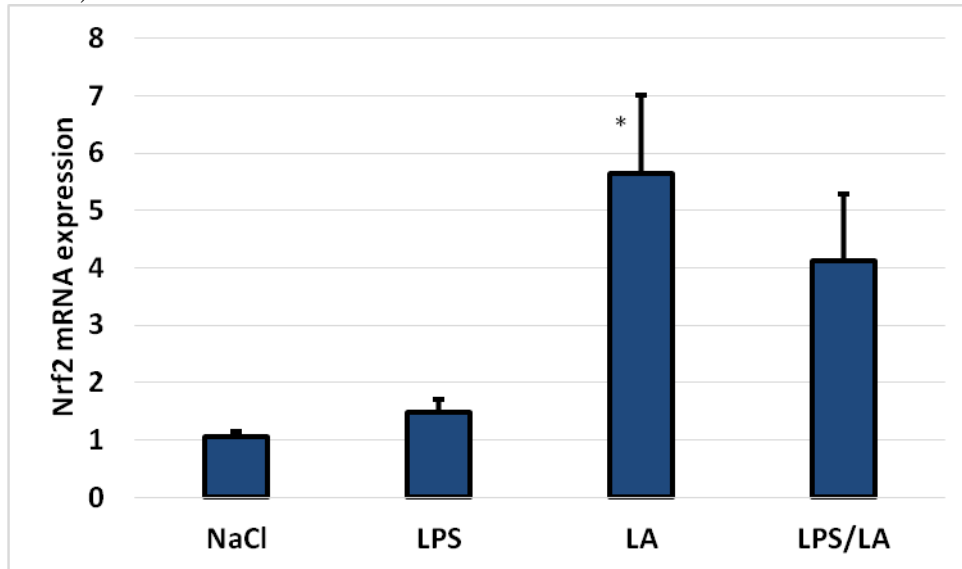
$p < 0,05$ vs LPS and LPS+LA

Discussion

Lipoic acid has a protective effect against oxidative stress in many tissues and organs, including the heart (Motawi et al., 2010), brain (Veskovic et al., 2015), liver and kidney (Petronilho et al., 2016), but its effects on skeletal muscle have not been clearly reported. Our present findings demonstrate that administration of α-lipoic acid can protect cells of skeletal muscle against oxidative stress induced by LPS.

Injection of LPS alone was seen to result in the development of oxidative stress, indicated by a significant increase in skeletal muscle lipid peroxidation and H_2O_2 concentration and a decrease in the free-SH group content of skeletal muscle. Administration of LA after the LPS challenge resulted in an increase in the total -SH group concentration, and a decrease in the degree of lipid peroxidation and H_2O_2 concentration in the skeletal muscle compared with the LPS groups. This was accompanied by the activation of Nrf2 after injection with LA. Our results suggest that LA may protect skeletal muscle against oxidative stress through up-regulating Nrf2 activity.

Nrf2 is a transcription factor that plays an important role in the protective molecular response to oxidative stress in cells (Zhong et al., 2013).

This factor, through binding to antioxidant response elements (AREs), induces expression of antioxidant enzymes that protect against oxidative damage. In the absence of oxidative stress, Nrf2 is retained in the cytosol by the actin-binding protein Keap1. However, under conditions of oxidative stress, Nrf2 is released from Keap1 and moves to the nucleus to induce expression of antioxidant enzymes.

Numerous studies based on mice lacking the Nrf2 gene confirm the key role played by Nrf2 in protecting against oxidative stress (Senger et al., 2016).

Skeletal muscle tissue is particularly vulnerable to oxidative stress, and greater oxidative damage, since it requires large amounts of oxygen to function properly, discuss the development of such inflammation in skeletal muscle tissue (Park et al., 2012).

The response of cells to excessive ROS involves the activation of multiple signaling pathways, including the Nrf2 pathway and nuclear factor-κB, leading to the up-regulation of numerous pro-inflammatory mediators, such as TNF-α, IL-1, IL-1β, and IL-6.

A recent study found that Nrf2 deficiency is associated with the dysregulation of skeletal muscle cytoprotective mechanisms upon induced oxidative stress (Miller et al., 2012).

Many studies have focused on the influence of LA on the Nrf2 pathway.

In rat liver, LA induced an early nuclear translocation of Nrf2 and activated antioxidant enzymes. Treatment with LA reversed the oxidative damage induced by palmitic acid (PA) in hepatocytes, and prevented obesity-induced oxidative stress in rat liver (Valdecantos et al., 2015).

In another study, the protective effect of LA was mediated not only through the enhancement of Nrf2 transcriptional activity, but also via the modulation of nuclear factor kappa B, cyclooxygenase-2, NADPH, and interleukin 17 in local and systemic damage in mice associated with ulcerative colitis (Trivedi et al., 2013).

Similar results were found in BEAS-2B human bronchial epithelial cells. Nrf2 production and its translocation from the cytoplasm to the nucleus were promoted by LA. These results showed that LA can protect human bronchial epithelial cells from paraquat (PQ)-induced ROS stress (Kim et al., 2013).

Other authors have examined the effect of LA on the expression of Nrf2-responsive genes of HepG2 cells exposed to arsenic (As^{3+}). LA was found to down-modulate the response mediated by Nrf2 and provide protection to As(3+) exposed HepG2 cells (Huerta-Olvera et al., 2010).

Several studies have shown that LA has a neuroprotective effect by promoting the translocation of Nrf2 to the nucleus. For example, LA has been found to have a neuroprotective effect against the death of the retinal neuronal RGC-5 cell line induced by oxidative stress (Koriyama et al., 2013).

Compounds that can activate the Nrf2 pathway, referred to as *Nrf2 activators*, are known to bestow beneficial health effects in diseases, involving oxidative stress.

As observed in the present study, these studies demonstrate that the protective effect of LA against oxidative stress can be mediated by the activation of the Nrf2 pathway. This activation offers beneficial effects in some diseases characterized by the overproduction of ROS.

Conclusions

The results indicate that LA treatment effectively protected the skeletal muscles against endotoxin-induced oxidative stress. This effect of LA is mediated through Nrf2 pathways.

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