SEVERE CONDITION OF ACIDOCIS IMPAIRS COX-2 AND HSP70 EXPRESSION IN LPS-STIMULATED J774.A1 MACROPHAGES.

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Abstract

Tissue acidosis is an hallmark of inflammatory, ischemic and tumoral processes and although it remains uncertain whether there is a true cause-effect relation between acidosis and adverse clinical outcomes it remains a powerful marker of poor prognosis in critically ill patients.

Dysregulation of host defence mechanisms and in particular of the immuno-inflammatory responses occurs during sepsis. Sepsis is always associated with acidosis conditions and is able to alter inflammatory mediators release and thus the immune response. Patients with severe sepsis and septic shock exhibit a complex metabolic pattern of acidosis at intensive care unit admission, caused predominantly by hyperchloremic acidosis, which was more pronounced in non survivors. In this study, conducted in vitro, we analyzed the effects of N-acetylcysteine (NAC), a molecule with antioxidant activity, during the inflammatory process, in acidosis conditions, in J774A.1 murine/macrophages stimulated with Lipopolysaccharide from *E.coli* (LPS). Our results show that NAC, in hyperchloremic acidosis conditions, reduces cyclooxygenase-2 (COX-2) and heat shock protein 70 (Hsp70) expression without significantly affecting changes in the constitutive isoform Heat shock protein 90β (Hsp90β) expression in LPS-treated macrophages .

Our data report a reduced inflammatory response exerted by NAC in hyperchloremic acidosis conditions indicating that the use of NAC during inflammation further impairs immune response associated to acidosis associated disease, as septic shock.

Keywords: N-acetylcysteine, macrophages, acidosis, LPS

Introduction

Systemic acidosis means an increase of the hydrogen ion concentration of any fluid or tissue with a consequent lowering of the pH. There are different types of acidosis: diabetic ketoacidosis, caused by a buildup of ketones [1]; hypercapnic acidosis; lactic acidosis [2,3]; respiratory acidosis, that is a result of increase in arterial PCO_2; and renal acidosis [4], caused by kidney malfunction, and metabolic acidosis [5].

Metabolic acidosis appears as an acute or chronic process that could have adverse effects on cellular functions and may contribute to the increase in morbidity and mortality [6,7]. Most of the acute toxic effects of metabolic acidosis occur on the cardiovascular system and consist of decreased cardiac output, arterial vasodilation and subsequent development of hypotension [8]. Acute metabolic acidosis is associated to states of mental confusion [9], changes in the affinity of hemoglobin for O2 [10] and insulin resistance [11]. In this pathological state, interleukins production is stimulated by macrophages, while lymphocyte function is suppressed leading to in an impairment of immune response and an increase in inflammation [12].

Sepsis is associated to a number of clinical manifestations, including acidosis. It was shown that the acid-base balance disorders encountered in many patients with sepsis significantly alter inflammatory mediators release [13], including cytokines, chemokines, and eicosanoids, often resulting in detrimental effects to the host [14,15]. Patients with severe sepsis and septic shock exhibit a complex metabolic acidosis at intensive care units admission, caused predominantly by hyperchloremic acidosis, which was more pronounced in nonsurvivors patients [16]. Metabolic acidosis may reduce survival in sepsis through several mechanisms. Moreover it has been demonstrated the effects of decreased extracellular pH on the synthesis and release of inflammatory mediators, such as nitric oxide (NO) [17] and Tumor Necrosis Factor-α (TNF-α) [18].

Previous animal and human studies [19,20] demonstrated elevated levels of prostanoids in both experimental and clinical sepsis syndrome. Prostaglandins E (PGE2) is one of the most potent and inducible of the prostanoids produced in states of inflammation. PGE2, produced by the metabolism of arachidonic acid by the enzyme cyclooxygenase (COX), is believed to be an important modulator of several of the observed events in sepsis. COX exists as two isoforms, COX-1 and COX-2. COX-1 is constitutively expressed, whereas COX-2 is expressed at low levels in most normal resting cells but is largely induced by a number of cytokines including TNF-α and inteleukin-1 (IL-1), mitogens or growth factors, lipopolysaccharide (LPS), and other inflammatory stimuli [21]. A marked upregulation of COX-2 occurs in synoviocytes, macrophages, and endothelial cells during stress and in inflammatory conditions such as sepsis.

Heat shock proteins (HSPs) are a highly evolutionarily conserved group of molecules; they play an important role in the host response to a wide variety of stresses, including infection, injury, oxidative damage, hypoxia, and thermal stress. HSP70 and HSP90 are the most extensively studied member of the family [22,23,24], and have been implicated in the pathophysiology of sepsis [22,23,24].

Sepsis has been associated with redox imbalance and oxidative stress and animal studies as well as prospective randomized clinical trials, have been providing increasing evidence in support of antioxidant therapies in sepsis [25,26]. Antioxidants can protect both extracellularly by scavenging toxic reactive oxygen species (ROS) and intracellularly by interrupting lipid peroxidation within the membrane. Antioxidant agents can also interfere early in inflammatory responses by blocking or modifying the signal transduction of inflammatory cytokines [27, 28]. N-acetylcysteine (NAC is an important cellular antioxidant that promotes detoxification, acts directly as free radical scavenger and decreases inflammation in various diseases [29,30].

We previously reported NAC capability to reduce NO, inducible Nitric Oxide Synthase expression, ROS release and to alter cell cycle distribution in J774A.1 macrophages. The aim of this study has been to evaluate the activity of NAC on COX-2 and Hsp involved in inflammatory response in a model *in vitro* of LPS-stimulated J774A.1 murine/macrophages in condition of hyperchloremic acidosis.

Methods

Reagents

Unless stated otherwise, all reagents and compounds were purchased from Sigma Chemicals Company (Sigma, Milan, Italy). The N-acetyl-Lcysteine powder, stored at 2°-8°C, soluble in water, was dissolved just before use in cell culture medium and used at concentrations of 5-10-15 mM. The Lipopolisaccharide from *Escherichia coli* (LPS),

in lyophilized powder was diluted in the medium of treatment to obtain the concentration of 1 µg/ml. The sterile hydrochloric acid in 1.0 N solution was diluted to obtain the concentrations of 17.1 mM and 2.5 mM corresponding to values of pH 6.5 and 7.4 as previously reported [17].

Cell culture

J774A.1 murine monocyte macrophage cell line (American Type Culture Collection, Rockville, MD), was grown in adherent to Petri dishes in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% foetal calf serum (FCS), 25 mM HEPES, 2mM glutamine, 100 u/mL penicillin and 100 mg/mL streptomycin at 37°C in a 5% CO_2 atmosphere.

Western blot analysis for COX-2, Hsp70 and Hsp90β

COX-2, Hsp70 and Hsp90β protein expression was assessed by Western blot analysis as previously reported [31]. Macrophages J774A.1 were seeded in p60 plated (1.8×10^6/P60) and allowed to adhere for 24h. After pretreatment with medium at two different pH (pH 6.5 and pH 7.4), the medium was replaced with fresh medium and cells were stimulated with NAC (5-10-15 mM) alone or in combination with LPS (1µg/ml) for further 24 h. Cells were scraped off, washed with ice-cold phosphate-buffered saline (PBS), and centrifuged at 5.000 g for 10 min at 4°C. The cell pellet was lysed in a buffer containing 20 mM Tris hydrogen chloride (HCl; pH 7.5), 1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, 10 µg/ml leupeptin, 10 mM sodium fluoride, 150 mM sodium chloride, 10 mg/ml trypsin inhibitor, and 1% Tween-20. Protein concentration was estimated by the Bio-Rad protein assay using bovine serum albumin as standard. Equal amounts of protein (50 µg) were dissolved in Laemmli's sample buffer, boiled, and run on a SDS polyacrylamide gel electrophoresis (SDS-PAGE) minigel (8% polyacrylamide) and then transferred to hybond polyvinylidene difluoride membrane for 40 min. at 5 mA cm2 into 0.45 µm. Membranes were blocked for 40 min in PBS and 5% (w/v) nonfat milk and subsequently probed overnight at 4°C with rabbit monoclonal anti-COX-2 and anti-Hsp90β, or with mouse monoclonal anti-Hsp70 and anti-tubulin in PBS, 5% w/v non fat milk, and 0.1% Tween-20. Blots were then incubated with horseradish peroxidase conjugated goat with horseradish peroxidase conjugated goat antimouse

immunoglobulin (Ig)G (1:5.000) for 1h at room temperature. Immunoreactive bands were visualized using electro-chemiluminescence assay (ECL) detection system according to the manufacturer's instructions and exposed to Kodak X-Omat film. The protein bands of iNOS on XOmat films were quantified by scanning densitometry (Imaging Densitometer GS- 700 BIO-RAD U.S.A.). Data are normalized with tubulin expression, used as reference protein, and expressed as arbitrary densitometric units as previously reported [32].

Data analysis

Data are reported as mean±standard error mean (s.e.m.); reported values are of at least three independent experiments. Statistical analysis was performed by analysis of variance test, and multiple comparisons were made by Bonferroni's test. A P-value less than 0.05 was considered significant.

Results

Effect of NAC on COX-2 expression at different pH in LPS-stimulated J774A.1 macrophages

By Western blot analysis of cellular lysates we analyzed if NAC influences COX-2 expression. Murine macrophages cell line J774A.1 were incubated with NAC (5-15 mM) in combination with LPS (1 µg/ml) for 24h at two different condition of pH, pH 6.5 and pH 7.4. A significant induction in COX-2 expression was observed in macrophages treated with LPS alone (P<0.001 vs control; Fig. 1). In presence of NAC (5-15 mM) at pH 7.4 COX-2 resulted significantly inhibited only at the highest concentration used (15 mM, P <0.01 vs LPS alone; Figure 1) while a significant reduction in COX-2 expression at pH 6.5 were observed at NAC highest concentrations (10 mM, P <0.05; 15 mM, P <0.001 Vs LPS alone; Figure 2). Furthermore in hyperchloremic conditions NAC exerted a stronger inhibition on COX-2 respect to pH 7.4 at all tested concentrations. (P<0.001 vs LPS+NAC pH 7.4; Fig. 2).

Effect of NAC on Hsp70 expression at different pH in LPS-stimulated J774A.1 macrophages

By Western blot analysis of cellular lysates, obtained 24 h after stimulation of J774A.1 macrophages with LPS, we observed a significant increase in Hsp70 expression, a protein involved in inflammatory process, in both pH conditions (Fig. 3, 4). When NAC (5-10-15 mM) was added to J774A.1 macrophages simultaneously with LPS, at pH 7.4 and 6.5, NAC exerted an inhibitory effect on Hsp70 expression

only at the concentrations of 10 mM (P <0.001 vs LPS alone) and 15 mM (P <0.001 vs LPS alone; Fig. 3) at pH 7.4 and only at the highest concentration tested at pH 6.5 (15 mM, P <0.05 vs LPS alone; Fig. 4).

Effect of NAC on Hsp90β expression from LPS stimulated macrophages in conditions of hyperchloremic acidosis

By Western blot analysis of cellular lysates, obtained 24 h after stimulation of macrophages with LPS, we observed that NAC didn't induce any significant change in the expression of the constitutive isoform Hsp90β in both conditions of pH of the medium (pH 6.5 and pH 7.4; Fig. 5, 6), but only a slow increase at the concentration of 15 mM at pH 7.4 and 15 and 10 mM at pH 6.5.

Discussion

Sepsis is a common and life-threatening medical condition, and despite recent advances in antibiotic therapy and intensive care, remains the most common cause of death in intensive care units [33]. The prominent features of sepsis are largely caused by the dysregulations of systemic inflammatory responses and are characterized by the excessive accumulation of proinflammatory mediators, such as TNF-α, IL-1 [34], interferon-gamma (IFN-γ) [35] and NO [36]. Thus, overwhelming inflammation plays a crucial role in host response to septic challenge [37,38].

Marked oxidative stress is a result of the inflammatory responses associated with sepsis and may result in multiple organ damage. Normally, a complex system of interacting antioxidant defence systems are able to balance oxidative stress and prevents damage; but during sepsis, excessive production of reactive oxygen species may induce significant cytotoxicity to organs and contribute to the multiorgan system failure [37].Furthermore the depletation of the important intracellular antioxidant GSH exerts an inflammatory effect. The antioxidant drug NAC increases cellular glutathione (GSH) levels and has recently been shown the NAC anti-inflammatory effects in LPS-stimulated macrophages [17]. In order to further investigate this effect, in the present study we evaluated the effect of NAC on COX-2, Hsp70 and Hsp90β expression in LPS- stimulated J774A.1 macrophages under conditions of physiological pH (pH 7.4) and in hyperchloremic acidosis conditions (pH 6.5), obtained via acidification of the incubation medium

with HCl [38]. Our results indicate that NAC reduces COX-2, and Hsp70 expression without significantly affect Hsp90β expression in J774A.1 macrophages.

COX-2 is a well known pro-inflammatory enzyme triggered by agents as LPS. COX-2 is poorly expressed in normal conditions, but appears as the dominant form in the course of inflammatory processes and it is involved in macrophage response. In our experiments, LPS induced in J774A.1 macrophages a marked increase in COX-2 expression and NAC significantly inhibited COX-2 expression respect to LPS. This effect was more pronounced in acidosis condition (pH 6.5) respect to physiological pH 7.4, thus indicating the inhibitory effect of NAC in acidosis condition on this important mediator of inflammatory response.

The HSPs are a family of highly conserved proteins constitutively expressed in cells and in response to stress conditions. Hsp70 and Hsp90 are potent activators of the natural immune response and they can induce the production of pro-inflammatory cytokines by the monocyte-macrophage system and the activation and maturation of dendritic cells through the transduction pathways related to Toll-like receptors type 2 and type 4 [39,40]. Inducible Hsp70 is one of the most important HSPs for maintenance of cell integrity during normal cellular growth as well as physiopathological conditions. In our experiments we observed a significant increase in Hsp70 expression in both pH conditions (pH 6.5-pH 7.4, P <0.001 vs control) in LPS-stimulated J774A.1 macrophages. Treatment with NAC determines at pH 7.4 a significant reduction in Hsp70 expression at the concentrations of 10 mM and 15 mM (P <0.001 vs LPS) and only at the highest concentration tested (P <0.05vs LPS) for pH 6.5. These results regarding the reduced Hsp70 expression by NAC are well in according with previous studies reporting the effect of antioxidant substances on the Hsp70 expression [41]. The reduced Hsp70 expression at pH7.4 and not at pH 6.5 could be addressed to a macrophage protective response against acidosis.

The presence of NAC didn't significantly affect Hsp90β expression in macrophages in presence of LPS at both two different pH.

The modulation of macrophage functions during inflammation by NAC it has been previously reported [42, 43]. NAC capability in reducing inflammatory response have to be considered beneficial in presence of an excessive macrophage response, not proportionate to the nature of the stimulus; but on

the other side in some conditions could assume a negative value as indicated by our results. Considering that in disorders associated to hyperchloraemic acidosis, as sepsis, there is a reduction in macrophages immune response the use of NAC could be useful to further reduce macrophage activcation and thus worsen the patient's clinical conditions.

Acknowledgments

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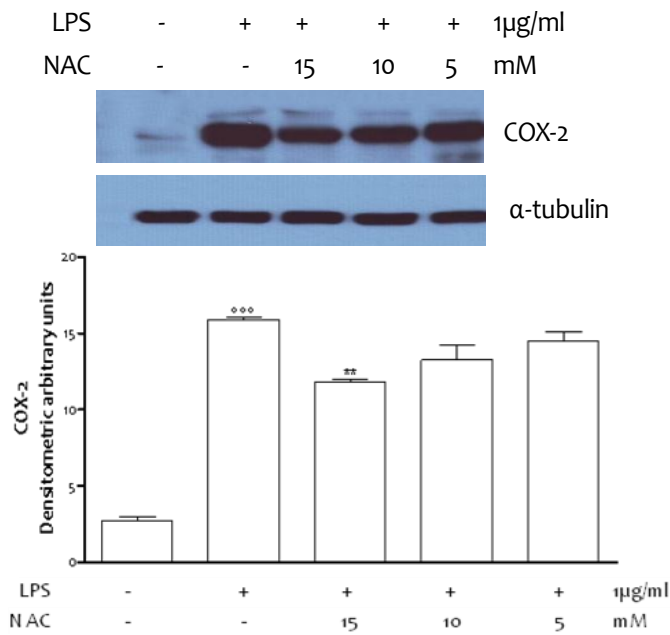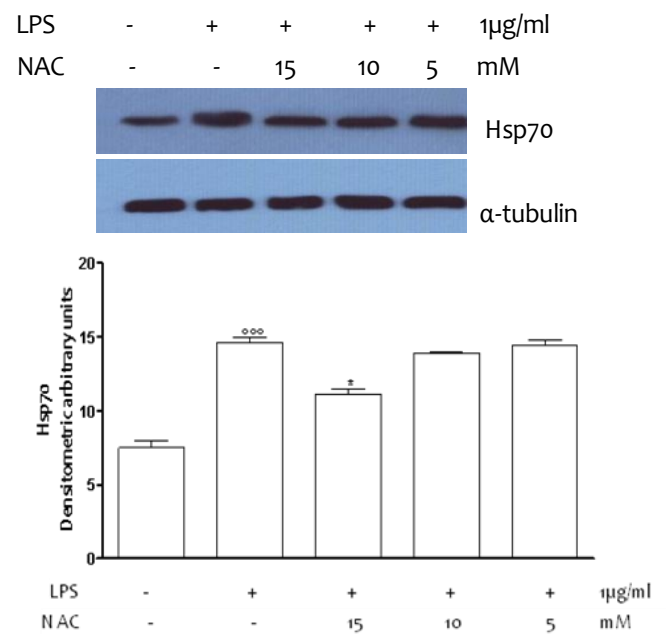
Fig. 1. Effect of NAC (5-10-15 mM) on COX-2 expression in macrophages J774A.1 treated with LPS (1 µg/mL) for 24 h at pH 7.4. The results obtained are expressed as mean ± SEM of 3 independent experiments. °°°P<0.001 vs CON, **P<0.01 vs LPS.
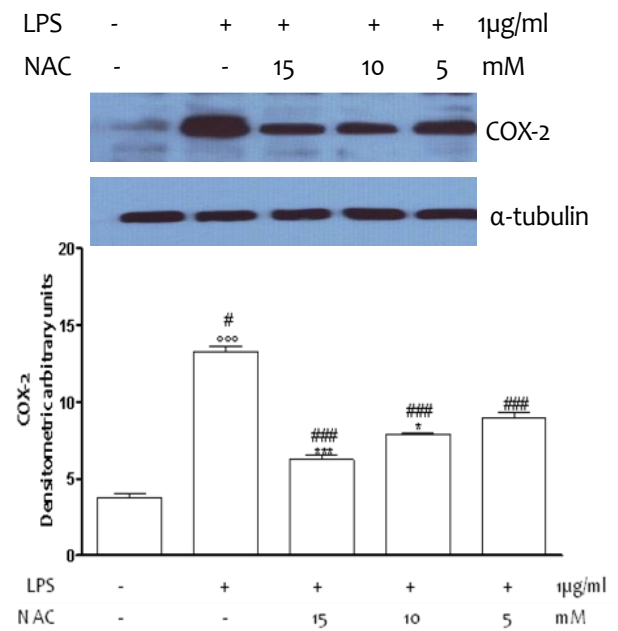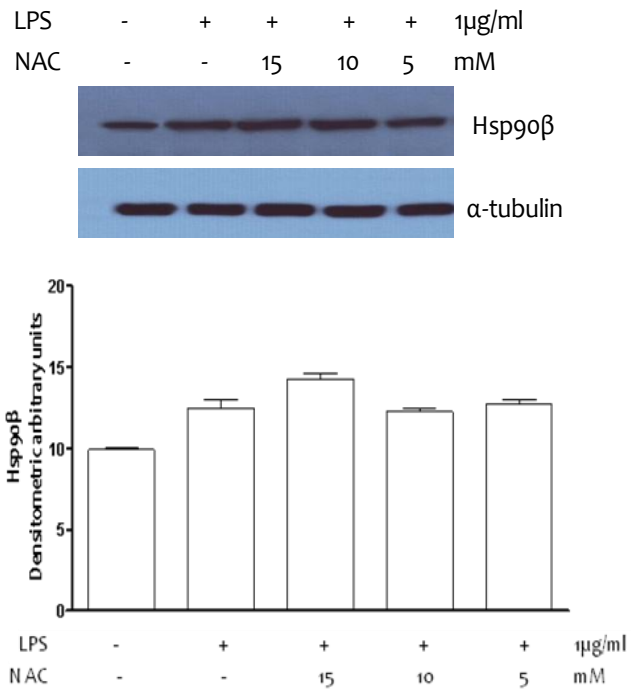

Fig. 2. Effect of NAC (5-15 mM) on COX-2 expression in macrophages J774A.1 treated with LPS (1 µg/mL) for 24 h at pH 6.5. The results obtained are expressed as mean ± SEM of 3 independent experiments. °°°P<0.001 vs CON, ***P<0.001 and *P <0.05, vs LPS; ### P<0.001 and # P<0.05, vs the same conditions at pH 7.4.
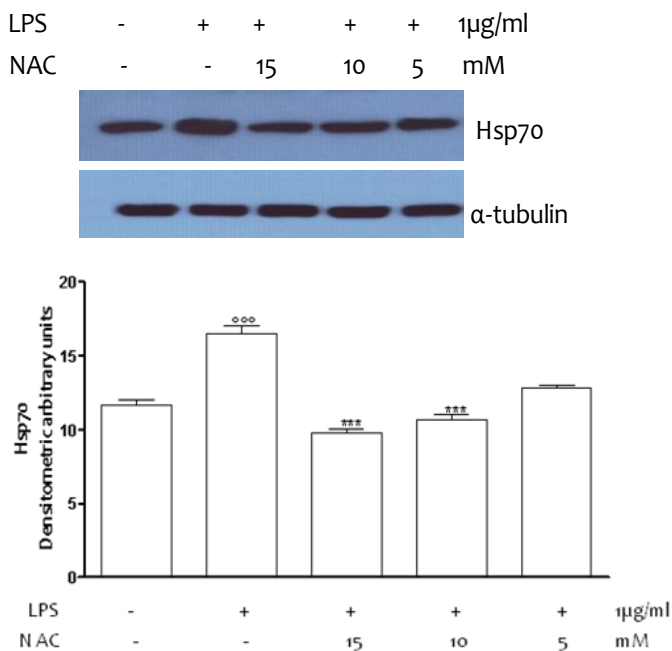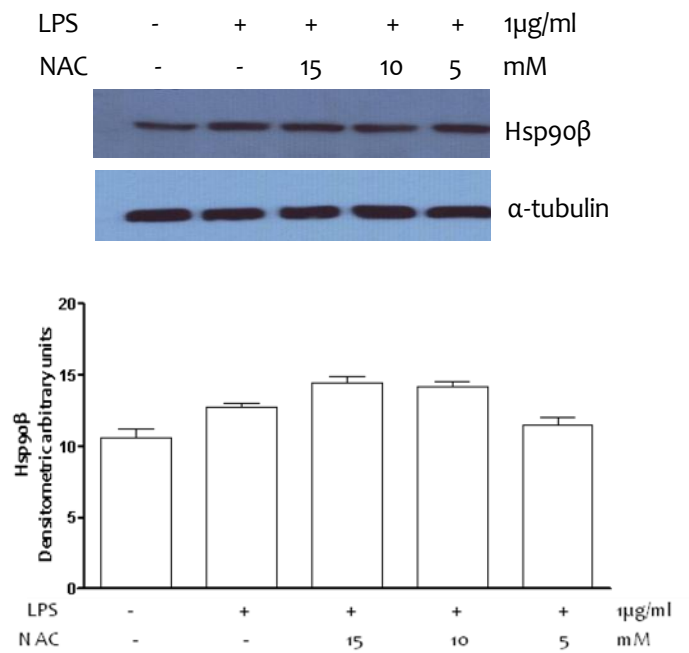

Fig. 3. Effect of NAC (5-10-15 mM) on Hsp**70** expression in J774A.1 macrophages-incubated with LPS (1 µg/mL) for 24 h at pH 7.4. The obtained results are expressed as mean ± SEM of 3 independent experiments. °°°P<0.001 vs CON, ***P<0.001 vs LPS.

Fig. 4. Effect of NAC (5-10-15 mM) on Hsp70 expression in J774A.1 macrophages incubated with LPS (1 µg/mL) for 24 h at pH 6.5. The obtained results are expressed as mean ± SEM of 3 independent experiments. °°°P<0.001 vs CON, *P <0.05 vs LPS.

Fig. 5. Effect of NAC (5-10-15 mM) on the expression of Hsp90β in LPS-stimulated co-incubated macrophages J774A.1 for 24 h at pH 7.4. The results obtained are expressed as mean ± SEM of 3 independent experiments.

Fig. 6. *Effect of NAC (5-10-15 mM) on the expression of Hsp90β in macrophages J774A.1 co-incubated with LPS (1 μg/mL) for 24 h at pH 6.5. The results obtained are expressed as mean ± SEM of 3 independent experiments.*