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N-acetylcysteine Improves In Vitro The Function of Macrophages from Mice With Endotoxin-induced Oxidative Stress

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Reactive oxygen species (ROS) and proinflammatory cytokines produced by immune cells cause the oxidative stress involved in septic shock induced by endotoxin. This oxidative stress can be controlled to a certain degree by antioxidants, which is specially important for a type of immune cell, i.e. the phagocyte, that uses ROS to kill microorganisms and needs antioxidants in order to support its functions. In a previous study we have observed changes in several functions of peritoneal macrophages from BALB/c mice with lethal endotoxic shock caused by intraperitoneal injection of Escherichia coli lipopolysaccharide (LPS) (100 mg/kg), which were associated with a high production of superoxide anion. N-acetylcysteine (NAC) is a thiolic antioxidant that improves the immune response, and we have observed that when administered intraperitoneally (150 mg/kg) at 30 min after LPS injection it counteracts the effects of LPS on macrophages and lymphocytes. In the present work, we have studied the in vitro effect of several concentrations of NAC (0.001, 0.01, 0.1, 1 and 2.5 mM) on the following functions: adherence to substrate, chemotaxis, ingestion of particles, ROS production and the release of tumor necrosis factor (TNFα) of peritoneal macrophages from BALB/c mice at 2, 4, 12 and 24 h after LPS injection. The results show that the administration of NAC (especially at 0.1 mM) decreases raised adherence, ingestion, ROS production and TNFα levels in macrophages from animals injected with LPS, bringing these functions to values near those of control animals. These effects which seem to be linked to a modulation of NF-κB, suggest that the improvement of immune functions observed in previous work after injection of NAC to animals with endotoxic shock could be due to a direct action of this thiol antioxidant on immune cells.

Keywords: N-acetylcysteine; Macrophage functions; Oxidative stress; Endotoxin; Mice

INTRODUCTION

Lipopolysaccharide (LPS), Gram-negative bacterial endotoxin, causes various pathophysiological changes that lead to septic shock, which is a systemic reaction to severe infection resulting in multiple organ failure and high mortality.[1] The generation of an immune response to endotoxins involve the activation of effector cells, mainly phagocytes, and the subsequent production of cytokines such as tumor necrosis factor (TNFα), and reactive oxygen species (ROS).[2] However, if these immune cells are excessively stimulated they can cause host cell damage and particularly endothelial injury.[3] Macrophages are ubiquitous mononuclear phagocytes in mammalian tissues, and the peritoneal macrophages, which are representative of other macrophage populations,[4] are easily available in mice in greater amounts than blood phagocytes (monocytes or neutrophils). The sequence of functions carried out by macrophages in the course of their phagocytic process is: adherence to tissue substrates, mobility directed to a chemical gradient from infectious foci (chemotaxis), ingestion of foreign agents and destruction of those agents by ROS production. This process represents the start of other biological activities that comprise the whole spectrum of the immune response. ROS injure tissues through peroxidation of membrane lipids, breakage of DNA strands, alteration of amino acids and disruption of

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cellular metabolism.[5] ROS and TNFα have provoked considerable interest in recent years as major contributors to endotoxin-induced tissue injury, which is associated with high mortality.

In a previous study, we have observed changes in the above mentioned functions of peritoneal macrophages from BALB/c mice with lethal endotoxic shock, especially a high production of superoxide anion.[6] Although several studies have demonstrated that pretreatment of animals with agents known to augment the cellular antioxidant defense system and to neutralize ROS can attenuate the shock state,[7] the use of antioxidants in the treatment of sepsis provides contradictory results.[8] However we have observed previously that the antioxidant N-acetylcysteine (NAC) counteracts *in vivo* the effects produced by LPS on murine peritoneal macrophages[9] and lymphocytes,[10] delaying the mortality of these animals several hours.

NAC is a compound that increases the pool of glutathione, which is an important cellular antioxidant[11] decreased during endotoxic shock.[12] NAC is easily absorbed, is useful to immune cells,[13] and influences several steps of the phagocytic process.[14] NAC inhibits the activation of the nuclear transcription factor NF-κB produced by LPS,[15] which could result in a decrease of TNFα synthesis, a key cytokine involved in endotoxic shock with effect on different steps of the phagocytic process.[16] Therefore, the aim of the present study was to investigate if the improvement of macrophage functions found in our previous work after an intraperitoneal injection of NAC in mice with endotoxic shock, could be due to a direct action of this antioxidant on the immune cell.

MATERIALS AND METHODS

Animals

Female BALB/c mice (*Mus musculus*), aged 24 ± 2 weeks (Criffa España; Barcelona, Spain), were maintained at a constant temperature of 22 ± 2°C on a 12 h light/dark cycle and fed Sander Mus (Panlab; Barcelona, Spain) and water *ad libitum*. The animals used did not show any sign of malignancy or other pathological processes. Mice were treated according to the guidelines of the European Community Council Directives 86/6091 EEC. Although in previous studies we observed that the oestrous cycle phase of the female mice has no effect on the kind of experimental assay carried out in the present study, all females used were at the beginning of dioestrous.

Materials

Lipopolysaccharide (*Escherichia coli* 055:B5), NAC, f-met-leu-phe, latex beads, nitroblue tetrazolium (NBT), EGTA, phorbol myristate acetate (PMA), HEPES, KCl, NaCl, EDTA, dithiothreitol, phenylmethylsulfonyl fluoride, aprotinin, leupeptin, pepstatin, NaN_3, Nonidet P-40, albumin, glycerol, SDS-polyacrilamide, Tris, glycine, Tween 20, 1,4-dithioerythritol and L-glutamine were purchased from Sigma (St. Louis, MO, USA). Culture plates were obtained from Steriling (Teddington, UK) and from Corning Glass (Corning, NY, USA). Filter from Millipore (Bedford, MA). Trypan blue was obtained from Merck (Darmstadt, FRG), and Diff-Quick pack for plate staining from Dade-Grifols. NAC and LPS were dissolved in phosphate-buffered saline (PBS) solution. Tumor necrosis factor alpha (TNFα) immunoassay was obtained from R&D Systems (Minneapolis, USA). RPMI 1640 medium, calf serum, penicillin and streptomycin were purchased from Gibco (Paisley, Scotland, UK). 2′, 7′-dichlorofluoresceine (DDF-DA) was obtained from Molecular Probes (Eugene, Oregon, USA). Antibodies against p50 and p65 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Dulbecco's modified Eagle's medium was purchased from Bio Whitaker (CA, USA).

Raw 264.7 mouse macrophage cells (ATCC, Manasses, VA) were used for the western blot test because a high amount of cells are needed for this method and those cells respond in the same fashion that peritoneal macrophages in the presence of LPS.[17] The cells were cultured in Dulbecco's modified Eagle's medium, supplemented with 2 mM L-glutamine, 100 units/ml penicillin, 10 mg/ml streptomycin and heat-inactivated (56°C, 30 min) 10% calf serum. Nonadherent cells were removed by aspiration and washed twice with Dulbecco's modified Eagle's medium.

Experimental Protocol

Oxidative stress was induced by intraperitoneal injection (i.p.) of *Escherichia coli* LPS (055:B5), at a concentration of 100 mg/kg according to a lethal endotoxic shock model that can be induced in this[6] and other murine strains.[18] Each experimental animal received this concentration of LPS in a volume of 100 μl of PBS between 9:00 and 10:00 a.m, and the control animals received the same volume of PBS. In this study, we have observed that LPS injection (100 mg/kg) caused a mortality of 100% at 26–30 h after LPS injection.

Collection of Peritoneal Exudate Cells

At 2, 4, 12 and 24 h after injection, peritoneal suspensions were obtained by a procedure previously described.[19] Briefly, 3 ml of Hank's solution adjusted to pH 7.4 were injected intraperitoneally, then the abdomen was massaged and

the peritoneal exudate cells were collected allowing recovery of 90–95% of the injected volume. Resting macrophages, identified by morphology and nonspecific esterase staining, were counted and adjusted in Hank's solution to 5×10^5 macrophages/ml. Cellular viability was routinely measured before and after each experiment by the trypan-blue exclusion test. In all cases the viability was higher than 95%. All incubations were performed at 37°C in a humidified atmosphere of 5% CO_2. The concentrations of NAC used to assay the *in vitro* effect on different functions of macrophages from the LPS group and control animals, were 0.001, 0.01, 0.1, 1 and 2.5 mM, since they had previously shown an effect *in vitro* on macrophages.[14]

Assay of Macrophage Functions

The quantification of substrate adherence capacity was carried out by a method previously described.[6,9,20] Aliquots of 200 μl of peritoneal suspension were placed in eppendorf tubes, and 20 μl of the different concentrations of NAC or 20 μl of Hank's solution (control samples) were added to the macrophages obtained from control animals or animals injected with LPS. At 20 min of incubation, 10 μl from each sample were removed after gently shaking to resuspend the sedimented cells, and the number of nonadhered macrophages was determined by counting in Neubauer chambers (Blau Brand, Germany) in an optical microscope (40× magnification lens). The adherence index, AI, was calculated as follows:

$$AI = 100 - [(\text{macrophages/ml supernatant})/(\text{macrophages/ml original sample})] \times 100$$

Chemotaxis was evaluated according to a modification[20] of the original technique described by Boyden[21] consisting basically on the use of chambers with two compartments separated by a filter with a pore diameter of 3 μm. Aliquots of 300 μl of peritoneal suspensions obtained from control animals or injected with LPS were deposited in the upper compartment with 30 μl of the different concentrations of NAC or with Hank's solution in the control samples. Aliquots of 400 μl of f-met-leu-phe (10^{-8} M), a well-known chemoattractant for macrophages,[22] were placed into the lower compartment. The chambers were incubated for 3 h (time chosen as optimum after previous experiments), and then the filters were fixed and stained. The chemotaxis index was determined by counting in an optical microscope (100× magnification lens) the total number of macrophages in the lower face of the filter.

Phagocytosis assay of inert particles (latex beads) was carried out according to a technique previously described.[19] Aliquots of 200 μl of the different peritoneal suspensions were incubated in culture plates for 30 min. The adhered monolayer was obtained and then 200 μl of Hank's solution and 20 μl of latex beads (1.09 μm of diameter) were added, as well as 20 μl of the different concentrations of NAC or Hank's solution (control samples). After 30 min incubation the plates were washed, fixed and stained, and the number of particles ingested by 100 macrophages was determined by counting in an optical microscope (100× magnification lens).

The superoxide anion production was evaluated assessing the capacity of this anion to reduce, in an equimolecular reaction, the nitroblue tetrazolium (NBT) giving a formazan measured by spectrophotometry at 525 nm.[23] The NBT reduction test was carried out following the method described by De la Fuente *et al.* in 1991.[20] Briefly, aliquots of 250 μl of peritoneal suspension were mixed with 250 μl of NBT solution (1 mg/ml in Hank's) and 50 μl of the different concentrations of NAC or Hank's solution (control samples), and either 50 μl of latex beads, in the stimulated samples, or 50 μl of Hank's, in the nonstimulated samples. After 60 min incubation in a bath at 37°C, the reaction was stopped, the samples were centrifuged, and the supernatants discarded. The intracellular reduced NBT was extracted with dioxan, and the absorbance of the supernatants was determined in a spectrophotometer at 525 nm. The data obtained were expressed as nmoles of NBT reduced per 10^6 macrophages by extrapolating from a standard curve of NBT reduced with 1,4-dithioerythritol.

The reactive oxygen species production was measured by flow cytometry using dichlorodihydrofluoresceine diacetate (DDF-DA) as a probe since it is oxidized in the cytoplasm by ROS to 2′, 7′-dichlorofluoresceine (DCF), which is a highly fluorescent compound. Aliquots of 200 μl of the peritoneal suspension (adjusted to 10^6 macrophages/ml) were centrifuged for 10 min at 1500 rpm and 4°C. The supernatants were discarded and the pellets were resuspended in 200 μl of Buffer A (Hank's medium without Ca^{2+} and Mg^{2+} and with EGTA 1 mM). The samples were incubated with 2 μl of DDF-DA (0.5 mM) for 15 min at 37°C. After incubation, 40 μl of phorbol miristate acetate (PMA) (positive control) and 40 μl of Buffer A were added to the stimulated and the control samples, respectively. For the *in vitro* effect of antioxidant, 40 μl of NAC at 0.1 mM were added. Samples were incubated for 15 min at 37°C and were then analyzed using a FACScan flow cytometer (Becton Dickinson, San Diego, USA). The results were expressed as fluorescence units (FU).

The concentration of mouse tumor necrosis factor alpha (TNFα), was determined on macrophage culture supernatants. Peritoneal cells were incubated with Hank's solution at a final concentration of 2×10^5 macrophages / 200 μl / well in 96 well plates for 1 h to allow macrophages to form a monolayer. Then, after two washes, 200 μl of RPMI-1640 medium without phenol red and with L-glutamine and 10% heat-inactivated (56°C, 30 min) calf serum, and 20 μl of NAC (0.1 mM) or Hank's solution were added. After 24 h incubation, plates were centrifuged at 1200 rpm for 10 min and TNFα production was quantified in the supernatants using a mouse TNFα (Endogen, Woburn, MA, USA) immunoassay with recombinant mouse TNFα, a minimum detectable dose of mouse TNFα of 10 pg / ml and a limit of procedure of up to 1500 pg / ml.

Western blot detection of nuclear extracts for p50 and p65 were prepared by the mini-extraction procedure of Schreiber *et al.* in 1989 with slight modifications.[24] The Raw 264.7 murine macrophages were plated at a density of 10^7 cells / well in 6-well plates and incubated for 2 h at 37°C with LPS (10 μg/ml), or LPS plus NAC (0.1 mM), or NAC (0.1 mM) or medium. After that, the cells were washed twice with ice-cold phosphate-buffered saline / 0.1% bovine serum albumin, and scraped off the dishes. The cell pellets were homogenized with 0.4 ml of buffer A (10 mM HEPES, pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 10 μg / ml aprotinin, 10 μg / ml leupeptin, 10 μg / ml pepstatin, and 1 mM NaN_3). After 15 min in ice, Nonidet P-40 was added to a final concentration of 0.5%, the tubes were gently vortexed for 15 s, and the nuclei were sedimented and separated from the cytosol by centrifugation at 12,000*g* for 40 s. The pelleted nuclei were washed once with 0.2 ml of ice cold buffer A, and the soluble nuclear proteins were released by adding 0.1 ml of buffer C (20 mM HEPES, pH 7.9, 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, 25% glycerol, 1 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 10 μg / ml aprotinin, 10 μg / ml leupeptin, 10 μg / ml pepstatin, and 1 mM NaN_3). After incubation for 30 min in ice, followed by centrifuging for 10 min at 14,000 rpm at 4°C, the supernatants containing the nuclear proteins were harvested. The protein concentration was determined by the Bradford method, and aliquots were stored at −80°C. Nuclear extracts containing 20–30 μg of proteins were subjected to reducing SDS-polyacrylamide gel electrophoresis (12.5%). After electrophoresis, the gel was electroblotted in a Tris–glycine buffer containing 40% methanol on a nitrocellulose membrane (Trans-blot, Bio-Rad). The membrane was blocked with TBS-T buffer (10 mM Tris, pH 8.0, 150 mM NaCl, 0.05% Tween 20) containing 5% milk powder, for 1 h at room temperature and then incubated with primary antibodies (rabbit anti-mouse IgG) against NF-κB p50 (1:1000) and NF-κB p65 (1:1000), in TBS-T containing 1% milk powder for 2 h at room temperature. The membrane was washed with TBS-T and incubated with the secondary antibody (goat anti-rabbit IgG conjugated to horseradish peroxidase) at 1:5000 dilution for 1 h at room temperature. After washing three times in TBS-T for 5 min each, and once in TBS for 5 min, the membrane was drained briefly and subjected to the enhanced chemiluminescence detection system (ECL, Amersham Pharmacia Biotech). The X-ray films were exposed for 5–20 min, and quantified by densitometry using NIH Image software.

Statistical Analysis

The data are expressed as the mean±standard deviation (S.D.) of the values from the number of experiments shown in the figures. The data were statistically evaluated by the U Mann-Whitney test for unpaired observations of nonparametric data as well as the Wilcoxon's test for paired comparison, $p < 0.05$ being the minimum significance level.

RESULTS

Figure 1A and B shows the adherence indexes (AIs) at 20 min incubation in the presence of different concentrations of NAC or Hank's solution of peritoneal macrophages from animals after 2, 4, 12 or 24 h of LPS or PBS injections. The AIs were increased significantly at 2, 4, 12 ($p < 0.001$) and 24 h ($p < 0.05$) after LPS injection. The increment shown in adherence indexes in cells from animals injected with LPS was attenuated by NAC with statistically significant differences at 2, 4 and 12 h with 0.1 mM, at 4 and 12 h with 0.01 mM ($p < 0.01$) and at 4, 12 and 24 h with 0.1 mM ($p < 0.05$) (Fig. 1A). In cells from control animals (Fig. 1B), the presence *in vitro* of 1 and 2.5 mM of NAC caused an increase ($p < 0.001$) of the AI.

The chemotaxis indexes of peritoneal macrophages obtained at 2, 4, 12 and 24 h after LPS or PBS injection and incubated in the presence of different NAC concentrations are shown in Fig. 2A and B. In the LPS group a highly significant decrement was seen at 2, 4 ($p < 0.001$) and 12 h ($p < 0.01$) after LPS injection in comparison to the PBS group. In cells from this LPS group, NAC increased the chemotaxis indexes at all concentrations and in general at all different times after LPS injection. At short times (2 and 4 h) the effects were most significant and 0.1 mM was the most effective concentration, with the values being similar to those of the control animals, whereas at 12 h the

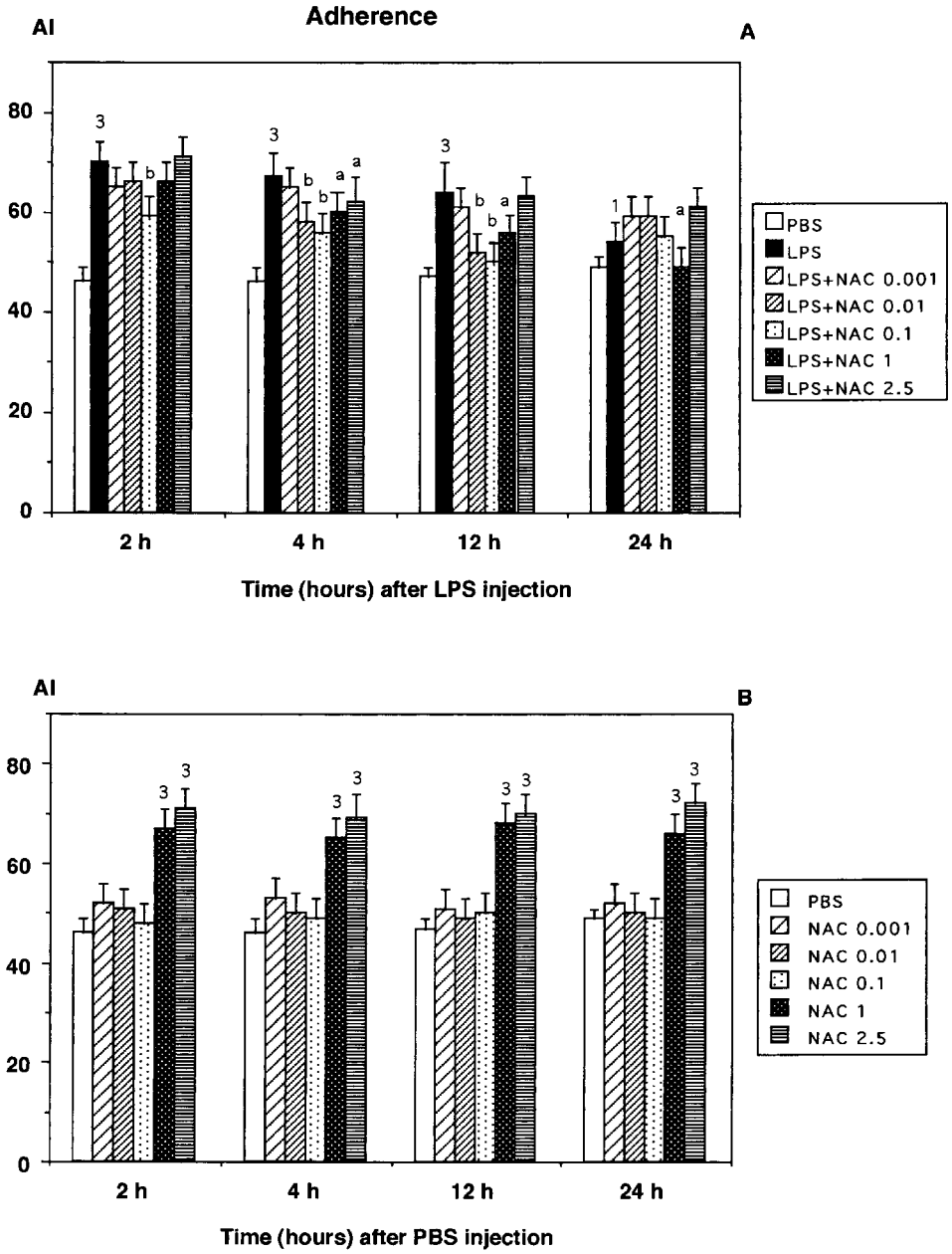

FIGURE 1 Adherence indexes at 20 min of incubation of peritoneal macrophages. (A) Lipopolysaccharide (LPS): cells from animals injected with 100 mg / kg of LPS; N-acetylcysteine (NAC). (B) Phosphate-buffered saline (PBS): cells from animals injected with PBS, control animals; N-acetylcysteine (NAC). The cells, in all cases were obtained at 2, 4, 12 and 24 h after injection. Each column represents the mean ± SD. of eight values corresponding to eight animals, each value being the mean of duplicate assays. $^{1}p < 0.05$ and $^{3}p < 0.001$ with respect to the corresponding values in the PBS group (controls). $^{a}p < 0.05$ and $^{b}p < 0.01$ with respect to corresponding values in samples from animals injected with LPS.

most effective concentrations were 1 and 2.5 mM. At 24 h, 0.1, 1 and 2.5 mM showed an increase ($p < 0.05$) of the chemotaxis index with respect to the controls. The presence of NAC in cells from control animals increased significantly the chemotaxis indexes ($p < 0.001$ with 2.5 and 1 mM, and $p < 0.01$ with 0.1 and 0.01 mM).

Figure 3A and B shows the phagocytosis indexes of murine peritoneal macrophages at 2, 4, 12 and 24 h after LPS or PBS injection and incubation in vitro in the presence of different NAC concentrations. In the LPS group a highly significant increment was seen at 2 h ($p < 0.01$) and at 4, 12 and 24 h ($p < 0.001$) after LPS injection in comparison to the PBS group. In macrophages from these animals, the presence in vitro of NAC caused a significant decrease at 2 h ($p < 0.05$) with 0.1 mM, at 4 h ($p < 0.01$) with 0.1, 0.01 and 0.001 mM, at 12 h ($p < 0.01$) with 0.01 and 0.1 mM, and at 24 h ($p < 0.01$) with 0.01 and 0.1 mM. The presence of NAC in cells from control animals significantly increased the phagocytosis indexes ($p < 0.01$ with 2.5, 0.1 and 0.001 mM, and $p < 0.001$ with 1 and 0.01 mM).

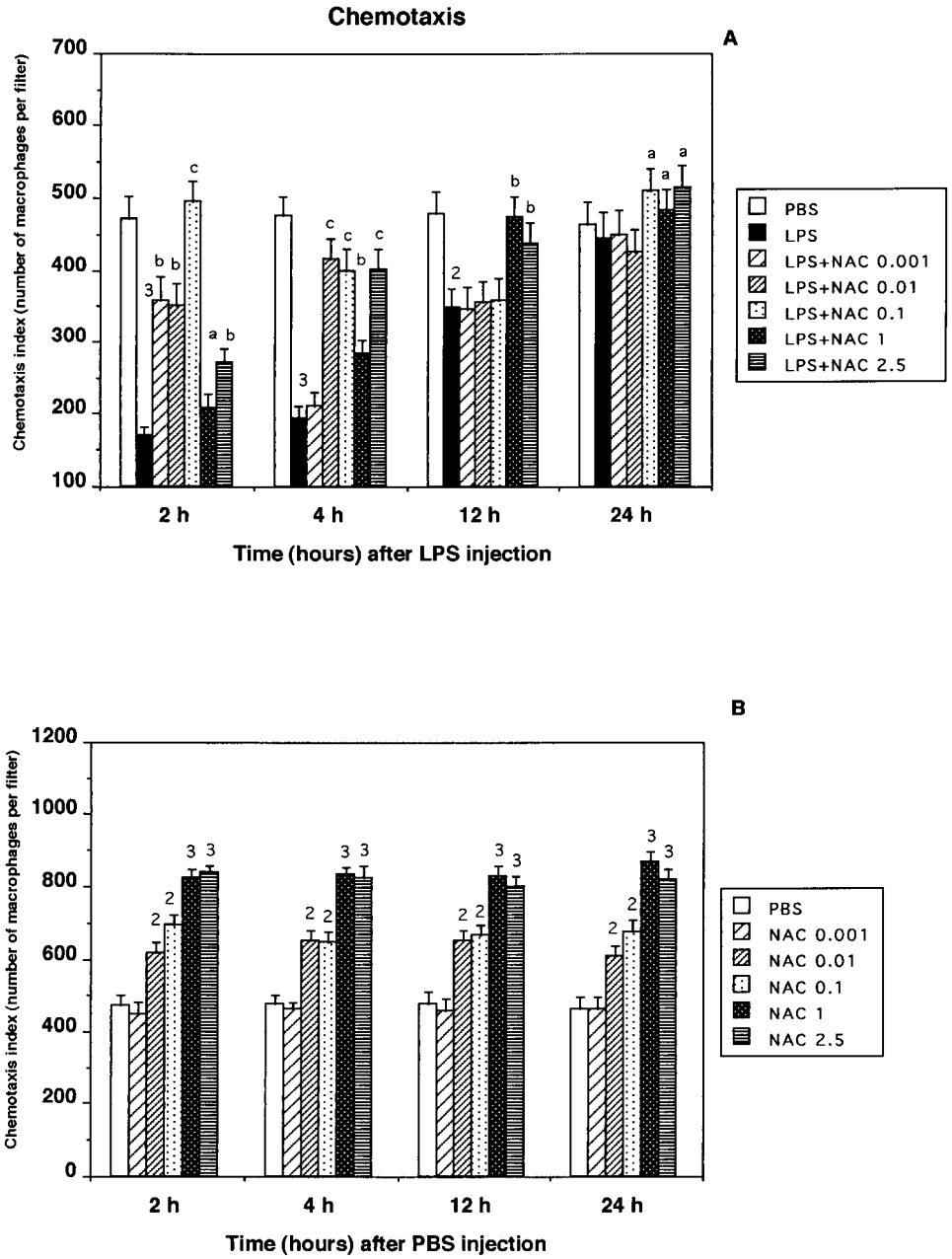

FIGURE 2 Chemotaxis indexes of peritoneal macrophages. (A) Lipopolysaccharide (LPS): cells from animals injected with 100 mg / kg of LPS; N-acetylcysteine (NAC). (B) Phosphate-buffered saline (PBS): cells from animals injected with PBS, control animals; N-acetylcysteine (NAC). The cells, in all cases were obtained at 2, 4, 12 and 24 h after injection. Each column represents the mean ± SD of eight values corresponding to eight animals, each value being the mean of duplicate assays. $^{2}p < 0.01$ and $^{3}p < 0.001$ with respect to the corresponding values in the PBS group (controls). $^{a}p < 0.05$ and $^{b}p < 0.01$ $^{c}p < 0.001$ with respect to corresponding values in samples from animals injected with LPS.

The values of anion superoxide production by peritoneal cells, measured by the NBT reduction test (expressed as nmoles / 10^6 cells) in nonstimulated (in the absence of ingestion material) and stimulated (in the presence of latex beads as ingestion material) samples, are shown in Fig. 4A and B) and Fig. 5A and B, respectively. The differences between groups were similar for both the stimulated and nonstimulated samples, although the values were always higher in the stimulated samples. At all times (2, 4, 12 and 24 h) superoxide anion production showed a highly significant increment ($p < 0.001$) after LPS injection although at 12 h the production of superoxide anion was lower that at the other times investigated. The presence of NAC in the cells from the LPS group (in the nonstimulated samples, Fig. 4A) decreased superoxide anion production with this effect being more significant with 0.1 mM of NAC at 4, 12, and 24 h after LPS injection, whereas 2.5 and 1 mM increased superoxide production at 2 h ($p < 0.05$). The presence of NAC in the cells from the LPS group (in the stimulated samples, Fig. 5A) also decreased

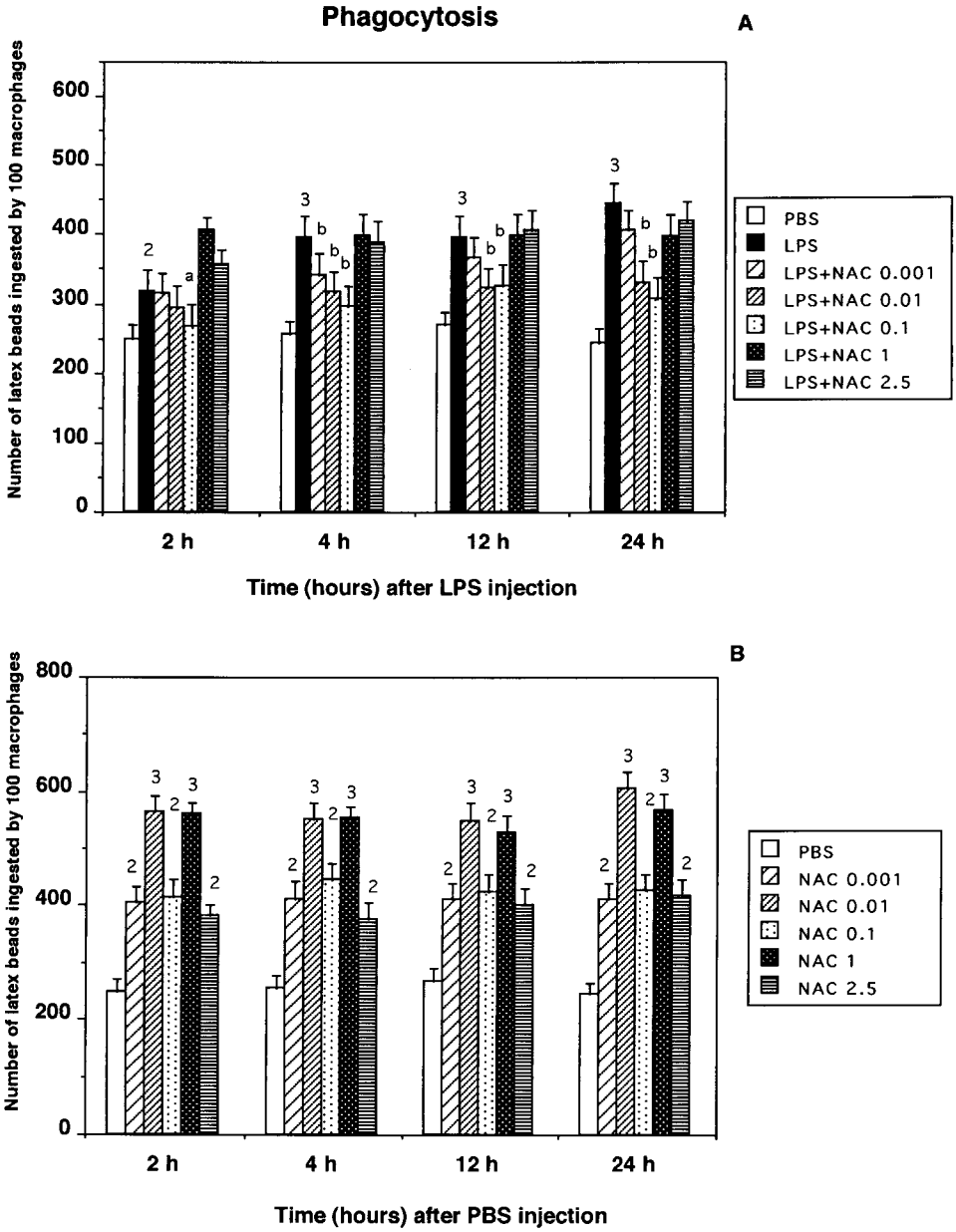

FIGURE 3 Number of latex beads ingested per 100 peritoneal macrophages. (A) Lipopolysaccharide (LPS): cells from animals injected with 100 mg/kg of LPS; N-acetylcysteine (NAC). (B) Phosphate-buffered saline (PBS): cells from animals injected with PBS, control animals; N-acetylcysteine (NAC). The cells, in all cases were obtained at 2, 4, 12 and 24 h after injection. Each column represent the mean ± SD of eight values corresponding to eight animals, each value being the mean of duplicate assays. $^{2}p < 0.01$ and $^{3}p < 0.001$ with respect to the corresponding values in the PBS group (controls). $^{a}p < 0.05$ and $^{b}p < 0.01$ with respect to corresponding values in samples from animals injected with LPS.

superoxide anion production, with 0.1 mM being the most effective concentration at all times after LPS injection. In cells from control animals (Figs. 4B and 5B) 0.1, 1 and 2.5 mM of NAC increased ($p < 0.01$) superoxide production.

ROS production by peritoneal macrophages from mice injected with LPS was higher than the control values at 4 and 12 h ($p < 0.05$), and at 24 h ($p < 0.01$) (Fig. 6). These values were decreased in the presence of NAC ($p < 0.05$), but in the control samples the values increased in the presence of NAC ($p < 0.05$). After stimulation with PMA, macrophages at 0 and 24 h after LPS injection increased their production of ROS ($p < 0.001$ and $p < 0.01$, respectively). This effect was decreased in the presence of NAC ($p < 0.001$) in the 0 h group, ($p < 0.05$) in the 4 and 12 h groups, and ($p < 0.01$) in the 24 h group.

The release of TNFα by peritoneal macrophages from mice injected with LPS was increased with respect to the control values (34 ± 10 pg/ml), at all the times studied, i.e.: 468 ± 38 ($p < 0.001$), 339 ± 43 ($p < 0.01$) and 289 ± 29 pg/ml ($p < 0.01$) at 2, 4 and 24 h, respectively. In the presence of NAC (0.1 mM) the values of TNFα released by peritoneal macrophages from mice injected with LPS were decreased

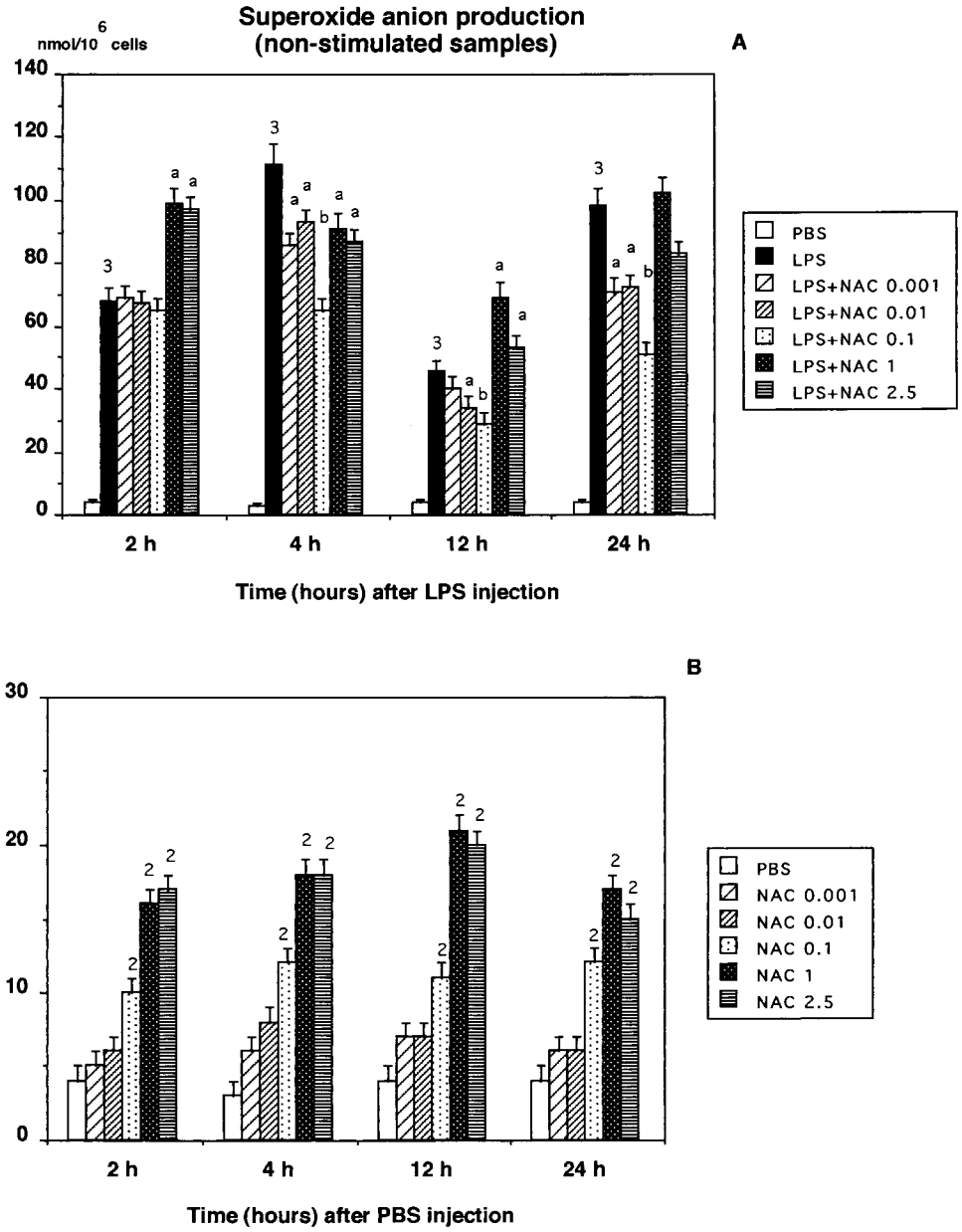

FIGURE 4 Superoxide anion production (nmol/10^6 cells) by peritoneal macrophages in nonstimulated samples. (A) Lipopolysaccharide (LPS): cells from animals injected with 100 mg/kg of LPS; N-acetylcysteine (NAC). (B) Phosphate-buffered saline (PBS): cells from animals injected with PBS, control animals; N-acetylcysteine (NAC). The cells, in all cases were obtained at 2, 4, 12 and 24 h after injection. Each column represents the mean±S.D. of eight values corresponding to eight animals, each value being the mean of duplicate assays. $^2p < 0.01$ and $^3p < 0.001$ with respect to the corresponding values in the PBS group (controls). $^ap < 0.05$ and $^bp < 0.01$ with respect to corresponding values in samples from animals injected with LPS.

at all times studied, i.e.: 146 ± 46 ($p < 0.001$), 86 ± 14 ($p < 0.01$) and 70 ± 8 pg/ml ($p < 0.01$) at 2, 4 and 24 h, respectively. In the presence of NAC (0.1 mM) the values of TNFα in macrophages from animals injected with PBS were similar (41 ± 6 pg/ml) to those of to the controls.

The levels of NF-κB complex (p50 and p65) were increased in presence of LPS (Fig. 7A and B). NAC 0.1 mM induced a decrease in nuclear p50 and p65 ($p < 0.01$) (Fig. 7A and B), which indicates an inhibitory effect of NAC on the nuclear translocation of p50 and p65 in cells with oxidative stress situation caused by LPS.

DISCUSSION

ROS have been implicated in the pathogenesis of Gram-negative sepsis, and oxidative stress has been suggested as an underlying damaging factor involved in the high mortality associated with endotoxic shock.[6,25] Macrophages, i.e. phagocytic cells with an essential role in the immune response of the host to inflammatory and infectious processes, are the major producers of mediators such as ROS and proinflammatory cytokines like the tumor necrosis factor (TNFα) involved in endotoxic shock.[26] Since peritoneal macrophages seem to play an important role in

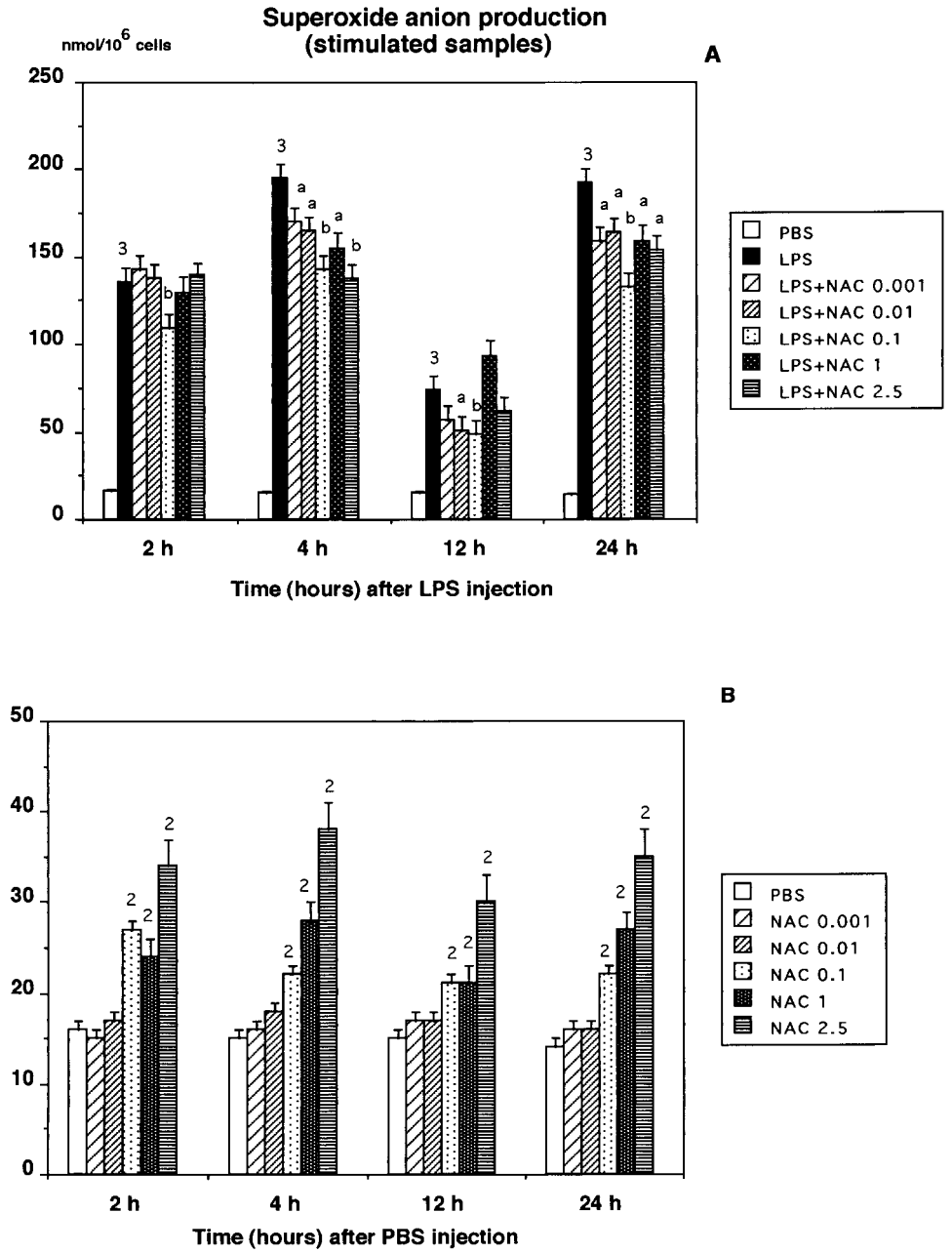

FIGURE 5 Superoxide anion production (nmol/10^6 cells) by peritoneal macrophages in stimulated samples (with latex beads). (A) Lipopolysaccharide (LPS): cells from animals injected with 100 mg/kg of LPS; N-acetylcysteine (NAC). (B) Phosphate-buffered saline (PBS): cells from animals injected with PBS, control animals; N-acetylcysteine (NAC). The cells, in all cases were obtained at 2, 4, 12 and 24 h after injection. Each column represents the mean ± SD of eight values corresponding to eight animals, each value being the mean of duplicate assays. $^2p < 0.01$ and $^3p < 0.001$ with respect to the corresponding values in the PBS group (controls). $^ap < 0.05$ and $^bp < 0.01$ with respect to corresponding values in samples from animals injected with LPS.

the pathogenesis of LPS, the attenuation of the toxic effects of those macrophage products can reduce the extent of LPS-induced injury. The antioxidant NAC is an oxygen free radical scavenger[27] and an inhibitor of the generation of TNFα through the inhibition of the transcription factor NF-κB,[27] as we have also observed in the present study for macrophage cells. Transcriptional regulation by LPS of the cytokine genes have been shown to involve a "NF-κB site."[28] NF-κB consists mostly of p50/p65 heterodimers, which are complexed to the inhibitor IκB in the cytoplasm of unstimulated cells. Stimuli such as LPS and proinflammatory cytokines induce the phosphorylation and degradation of IκB, followed by the release and subsequent nuclear translocation of the p50/p65 heterodimers, which bind to regulatory sequences in a variety of target genes.[29] The present study shows that NAC inhibits the levels of p50 and p65.

NAC shows stimulatory effects on the phagocytic process *in vitro*,[14] and it can increase the intracellular levels of glutathione, which acts as an

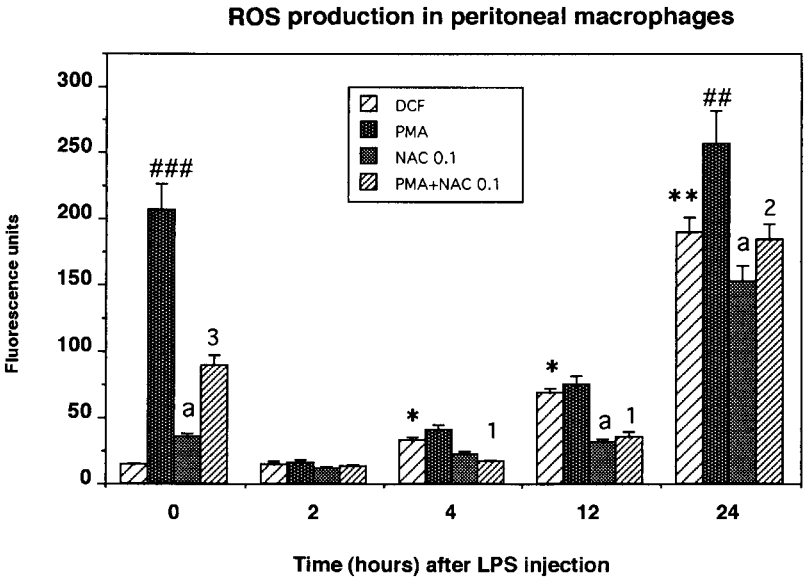

FIGURE 6 ROS production in peritoneal macrophages. DCF (cells from animals injected with 100 mg/kg of LPS at different times); phorbol myristate acetate (PMA), positive control, 50 ng/ml); N-acetylcysteine (NAC). In all cases, the cells were obtained at 0, 2, 4, 12 and 24 h after injection. Each column represents the mean ± SD of eight values corresponding to eight animals, each value being the mean of duplicate assays. $*p < 0.05$ and $**p < 0.01$ with respect to the corresponding values in the 0 group (controls). $^{\#\#}p < 0.01$ and $^{\#\#\#}p < 0.001$ with respect to the corresponding value in the DCF group. $^{a}p < 0.05$ with respect to the value in the corresponding control (DCF). $^{1}p < 0.05$, $^{2}p < 0.01$ and $^{3}p < 0.001$ with respect to the corresponding value in the PMA group.

antioxidant[30] and also stimulates the phagocytic function.[14] Therefore, the favorable in vivo effect of NAC observed in a previous study on this model of endotoxic shock[9] could be attributed either to NAC or to GSH increase. The present work performed in vitro suggests that NAC produces its effects on macrophages modulating the signaling pathways that lead to free radical production.

The first step of phagocytosis involves adherence of phagocytic cells to tissue substrate before migration of these cells to the site of inflammation. Phagocytic cell adherence to a smooth plastic surface is comparable to that taking place in animal tissues. LPS injection stimulates the adherence capacity of macrophages, which was previously observed in the endotoxic shock model.[6] The maximal stimulating effect of LPS was observed at 2 h after injection, which could be due to the increase of the adhesion molecule expression induced by ROS[31] or by TNFα.[32]. The increase of TNFα production after LPS stimulation, with a peak at 2 h, has been shown in this study as well as in a previous one[9] and it has also been observed by other authors.[18] The presence of NAC decreased the adherence capacity, especially at the 1, 0.1, and 0.01 mM concentrations, which could be explained by an homeostatic effect of this antioxidant, decreasing the TNFα levels through inhibition of NF-κB translocation. This homeostatic effect on adherence activity has been observed in our previous work on macrophages[9] and lymphocytes.[10] However, 2.5 mM of NAC did not decrease this function at 12 and 24 h after LPS injection, showing instead a pro-oxidant effect. In fact, we have observed in previous work that NAC administered in vivo at a concentration of 500 mg/kg had pro-oxidant effects while with 150 mg/kg this compound showed an antioxidant action.[9] Moreover, adherence was stimulated in cells from control animals with the highest concentrations of NAC (1 and 2.5 mM).

The chemotaxis capacity, which allows macrophages to reach the inflammation site, was significantly inhibited after LPS injection, as previously observed in the endotoxic shock model.[6] This is probably a consequence of the production of the migration inhibitory factor (MIF) in response to the increased TNFα.[33] Mobility was raised by NAC at all concentrations in cells from animals with endotoxic shock, reaching values similar to those of cells from control mice at concentrations of 0.1 and 0.01 mM. This effect is similar to that obtained in previous work using NAC in vivo on macrophages,[9] and lymphocytes.[10] This could be explained by the inhibitory effect of NAC on TNFα synthesis through its effect on NF-κB translocation, decreasing the levels of p50/p65. Chemotaxis was also stimulated by NAC in cells from control animals, which agrees with previous reports on in vitro[14] and in vivo research,[9] in which the presence or supply of NAC was associated with an enhancement of this function.

Phagocytosis of latex beads by macrophages from animals with endotoxic shock was increased at all times after LPS injection, which was previously observed in the endotoxic shock model.[6] Although NAC stimulated this macrophage function in vitro at all concentrations in macrophages from control animals, in agreement with a previous report,[14] a phagocytosis decline was observed when NAC at

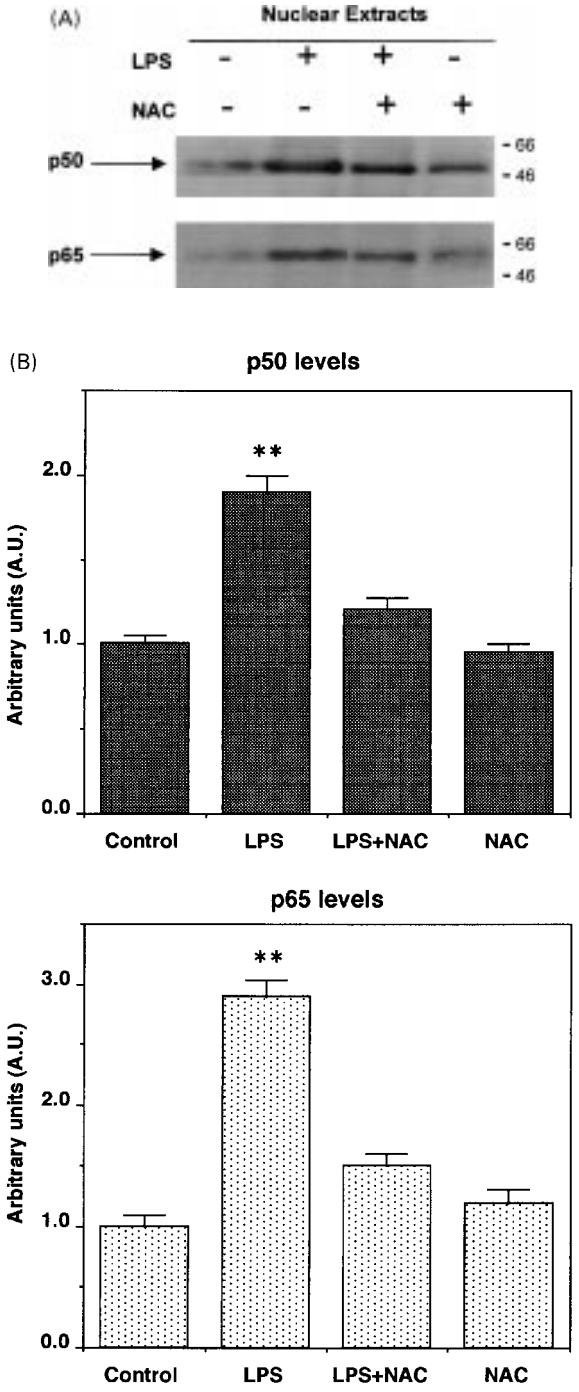

FIGURE 7 (A) Effect of NAC on p50 and p65 complexes. Raw 264.7 macrophages were stimulated with LPS, and NAC was added at a concentration of 0.1 mM. Control cultures were incubated with medium alone. Nuclear proteins were extracted 2 h after LPS stimulation. Western blot analysis was performed for p50 and p65 in the nuclear extracts. One representative experiment of three is shown. B. p50 and p65 levels. Each column represents the mean ± SD of three experiments, each value being the mean of duplicate assays. $**p < 0.01$ with respect to the corresponding values in the control group.

concentrations of 0.1 and 0.01 mM was added to cells from animals with endotoxic shock. This is probably caused by inhibition of TNFα synthesis, the result being similar to that obtained in our in vivo studies with NAC.[9]

The levels of ROS and superoxide anion were raised in macrophages from animals injected with LPS at all times studied, as we have observed previously in these cells and in lymphocytes[6,9,34] possibly as a result of the respiratory burst induced by LPS in those phagocytic cells.[35] An oxidant/antioxidant imbalance has been seen in a human study with septic shock, with total plasma antioxidants decreasing in patients.[36] ROS are necessary to eradicate infections, being useful if their production is controlled. Conversely, an uncontrolled production

of ROS causes tissue injury by peroxidation of lipid membranes, which can occur in endotoxic shock. In the present and previous studies[9,14] NAC increased ROS production in control cells, which represents an increment of the production of oxygen radicals during the process of foreign material digestion by macrophages. However, in this study NAC decreased ROS production in macrophages from mice with endotoxic shock at low doses (0.001, 0.01 and 0.1 mM). In agreement with this, Sprong *et al.*,[37] found a decreased oxidative stress in rats injected with LPS after injection of low doses of NAC.

In general, NAC counteracts both *in vivo*[9] and also, as it has been shown in the present work, *in vitro*, the negative effect induced by LPS during endotoxic shock on macrophage activity, thus playing an homeostatic role. The positive effect of NAC (especially at 0.1 mM) persistently suppresses LPS activation in macrophages, and suggests that the mechanism responsible for this involves more than mere quenching of free radical production, like has been observed in Kupffer cells.[38] Moreover, since NF-κB regulates the transcription of different monocyte/macrophage-derived proinflammatory genes, which are commonly associated with inflammatory and autoimmune disorders, the inhibition of the NF-κB transcriptional activity by NAC, decreasing the levels of p50/p65, could also explain its possible therapeutic effects.

Since an adequate immune response is essential for health preservation, the therapeutic efficacy of NAC in ameliorating LPS-induced phagocytic process changes suggests that treatment with this antioxidant may be useful in clinical conditions associated with oxidative stress in which endotoxins are a pathogenic factor.

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