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# α-Lipoic Acid Inhibits Endotoxin-stimulated Expression of iNOS and Nitric Oxide Independent of the Heat Shock Response in RAW 264.7 Cells

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The heat shock response protects against sepsis-induced mortality, organ injury, cardiovascular dysfunction, and apoptosis. Several inducers of the heat shock response, such as hyperthermia, sodium arsenite, and pyrollidine dithiocarbonate, inhibit NF-κB activation and nitric oxide formation. The antioxidant lipoic acid (LA) has recently been found to inhibit NF-κB activation and nitric oxide formation. We therefore tested the hypothesis that LA induces a heat shock response. To test this hypothesis, we determined whether exposure to LA affects expression of both heat shock protein 70 (HSP-70) and nuclear heat shock factor-1 (HSF-1) in lipopolysaccharide (LPS) stimulated macrophages. LA and hyperthermia attenuated LPS-induced increases in nuclear NF-κB, iNOS protein, and media nitrite concentrations. LPS and hyperthermia increased HSP-70 concentrations 8-fold and 20-fold, respectively. No effect of LA treatment alone on HSP-70 protein expression was detected. Likewise, no effect of LA on HSF-1 protein expression was detected. These data suggest that LA inhibits LPS-induced activation of iNOS in macrophages independent of the heat shock response.

*Keywords*: Endotoxin; HSP-70; HSF-1; Murine macrophages; NF-κB

## INTRODUCTION

The heat shock response represents a protective cellular response to stressful environmental or clinical conditions. Examples of stressors that induce a heat shock response include exposure to hyperthermia, sodium arsenite, heavy metals, ethanol, ischemia/reperfusion injury, and hemorrhagic shock. The protection afforded by a heat shock response relates to stress-induced expression of heat shock proteins that repair damaged proteins. Initiating the heat shock response involves binding of inactive nuclear heat shock factor-1 (HSF-1) monomers to form active homotrimers. These HSF-1 homotrimers bind to heat shock elements on the promotor regions of genes encoding heat shock proteins and molecular chaperones.[1] Transcriptional activation of heat shock proteins, such as HSP-70, requires phosphorylation of the HSF-1 trimer.[2]

Besides functioning in protein repair, the heat shock response affords protection against sepsis.[3,4] Activation of the heat shock response inhibits the nuclear translocation of NF-κB, an important regulator of inflammatory responses. Heat shock proteins inhibit upstream mediators of NF-κB nuclear translocation, such as IκK and IκB-α.[5,6] Activators of the heat shock response that inhibit inflammatory signaling during experimental sepsis include hyperthermia,[3,4] sodium arsenite[7,8] and the antioxidant pyrrolidine dithiocarbamate.[9]

α-Lipoic acid (LA) is a naturally occurring thiol antioxidant and an essential metabolic cofactor for pyruvate dehydrogenase and α-ketoglutarate dehydrogenase reactions.[10] LA is readily taken up by many cells and tissues and is reduced in mitochondria to the potent antioxidant,

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dihydrolipoic acid or DHLA. LA and DHLA act as a redox couple which can regenerate other natural antioxidants, such as glutathione, vitamin C, and vitamin E. Recent investigations have revealed potent antioxidant properties of LA which include scavenging of reactive oxygen species, regulating endogenous antioxidants, chelating metals, and repairing oxidative damage.[11–14] In addition to functioning as an antioxidant, LA inhibits the expression of a variety of inflammatory proteins, such as, inducible nitric oxide synthase (iNOS), cytokines (TNF-α, IL-1β, IL-6), chemokines (IL-8, MCP-1), and adhesion molecules (e-selectin, ICAM, VCAM).[15,16]

Like heat shock, LA inhibits nuclear translocation of NF-κB by preventing the activation of IκK and phosphorylation/degradation of IκB-α.[15] Interestingly, LA has been postulated to induce a heat shock response.[17] One proposed mechanism for an LA-induced heat shock response is that LA catalyzes the formation of intramolecular disulfides in certain signaling proteins that function as detectors of oxidants and/or electrophiles.[17] These disulfide containing signaling proteins promote activation of HSF-1 and heat shock genes. Whether LA induces a heat shock response has not been previously examined.

Here we examine the effects of LA on the NF-κB and heat shock signaling pathways in mouse macrophages (RAW 264.7 cells). We predicted that LA would induce a heat shock response because other inhibitors of NF-κB activation and NO synthesis (such as hyperthermia, sodium arsenite, and pyrrolidine dithiocarbamate) have been shown to induce a heat shock response. Specifically, we examined whether LA increases nuclear levels of heat shock factor (HSF-1) and subsequent expression of HSP-70. Such evidence would clarify the complex anti-inflammatory mechanisms triggered by LA and add further support for the safe use of LA therapy.



## MATERIALS AND METHODS

### Cell Culture

RAW 264.7 cells were grown in Dulbecco's modified Eagle's medium (DMEM, Life Technologies Incorporation, Grand Island, NY) supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin (Life Technologies Incorporation). The cells were grown on 10 cm diameter plates in a humidified chamber at 37°C using a mixture of 95% room air and 5% $CO_2$. Bacterial lipopolysaccharide (LPS) (*Escherichia coli* Serotype 0127:B8) (Sigma-Aldrich Company, St. Louis, MO) (100 ng/ml) was used to induce NO formation. Separate cultures were used to obtain whole cell protein and nuclear extracts. Additional cultures were grown on 96 well plates for determination of potential cytotoxicity of racemic R,L-alpha lipoic acid (LA) (Sigma-Aldrich Company, St. Louis, MO). Cultures were harvested for analysis after 18 h of exposure to LPS. This treatment period was chosen because preliminary studies revealed that iNOS protein expression in LPS stimulated RAW 264.7 cells peaks between 12 and 20 h of LPS exposure and declines after 24 h.

### Nitrite Concentration Response Curve to LA

Fresh LA (0.5 mol/l) stock solutions were prepared in 100% ethanol immediately before each experiment. The final concentration of ethanol in LA solutions added to culture media was 0.5% for all doses of LA. To control for potential effects of ethanol on outcomes, the concentration of ethanol in all other cultures not treated with LA was also adjusted to 0.5%. We treated near confluent cultures of RAW cells growing in 12 well plates with either 1, 3, 10, 30, 100, 300, 1000, 1500, and 3000 μmol/l LA 2 h prior to addition of 100 ng/ml LPS. To determine whether LA inhibits LPS-induced nitric oxide synthesis, we measured the sum of stable nitric oxide metabolites, nitrate ($NO_3^-$) and nitrite ($NO_2^-$), in culture media samples 18 h after exposure to LPS. Culture media was harvested for analysis nitrates using a colorimetric assay kit (Cayman Chemical, Ann Arbor, MI) that involves the Griess reaction. Nitrate concentrations in culture media were measured after reducing all nitrates into nitrite using nitrate reductase. The absorbances were read at a wavelength of 540 nm on a microplate reader (Powerwave X; Bio-Tek Instruments Incorporation, Vermont).

### Heat Shock and LA Treatment

Nearly confluent RAW cells growing on 10 cm diameter plates were randomly assigned to one of four treatment groups. Plates containing the control cultures were untreated and were maintained at 37°C. The LPS only group also consisted of plates of cells maintained at 37°C throughout the experiment. They received LPS (100 ng/ml) at the same time as HS and LA treated groups. A third group of plates were placed in a 42°C incubator for 60 min to elicit a heat shock response. They were then returned to the 37°C incubator and allowed to acclimate for 1 h. Afterward they received LPS (100 ng/ml) and were harvested 18 h later for total nitrite and nitrates and immunoblotting assays. Another group of cells were treated with α-lipoic acid (LA) for a period of 2 h. This occurred during the same 2 h period as the heat shock and acclimation periods of the cells that were heat shocked. These cells were subsequently treated with the same dose of LPS and harvested 18 h later. Two additional control groups were used in the experiment to examine the effects of different

treatments on expression of HSP-70. One group was treated with LA alone and the other treatment consisted of heat shock alone. The LA group received the vehicle solution for LPS and the HS group received the vehicle solution for LA.

### Immunoblotting Assay

Antibodies for heat shock protein 70 (HSP-70), heat shock transcription factor-1 (HSTF-1), and P65, were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). An antibody to inducible nitric oxide synthase (iNOS) was purchased from Transduction Laboratories Incorporation, Lexington, KY). For the detection of iNOS, HSP-70, HSF-1, P65, and IκB-α protein, cell cultures were harvested following treatment and protein extracts were prepared. The protein concentration of each sample was measured using a BCA protein assay kit (Pierce Chemical Company; Rockford, IL). Proteins were then separated by SDS-gel electrophoresis. For each protein investigated equal amounts of protein were loaded into wells of 7.5% *Tris*-glycine precast polyacrylamide gel (Bio-Rad Laboratories; Hercules, CA) and separated by application of a constant current using 50 V for 180 min with a Mini-Protean electrophoresis system (Bio-Rad).

After separating the proteins by electrophoresis, they were transferred from gels to nitrocellulose membranes (Bio-Rad) at 100 V constant current for 60 min. The nitrocellulose membranes were then blocked at room temperature for 60 min using a solution containing 5% non-fat dry milk in Tris buffered saline. After blocking, the membranes were incubated overnight at 4°C in the primary antibody solution (1:1000 dilution, murine monoclonal iNOS, IgG2a, antibody; 1:1000 dilution HSP-70, rabbit polyclonal antibody, HSF-1; 1:1000 dilution HSTF-1, rabbit polyclonal antibody; 1:1000 dilution P65, rabbit polyclonal antibody). Horseradish peroxidase conjugated sheep anti-mouse IgG antibody (1:1500 dilution; Amersham) or an HRP conjugated goat anti-rabbit IgG antibody (1:5000 dilution; Santa Cruz) were used as secondary antibodies. Bound antibody was detected using a chemiluminescence kit (ECL plus; Amersham) and high performance chemiluminescence film (Hyperfilm, Amersham). Densitometric techniques were then performed using Photoshop 7.0 software (Adobe Systems Incorporation, San Jose, CA) to quantify background-corrected protein band densities.

### Electrophoretic Mobility Shift Assay (EMSA) for NF-κB

Nuclear extracts were prepared using a NE-PER nuclear and cytoplasmic extraction kit (Pierce, Rockford, IL) according to manufacturer's instructions. EMSAs were performed using reagents contained in the Gel Shift Assay System from Promega Corporation (Madison, WI). The NF-κB consensus sequence (5′-AGT TGA GGG GAC TTT CCC AGG C-3′) described by Lenardo and Baltimore[18] was provided in the Gel Shift Assay kit. The consensus sequence for the heat shock response element (5′-GCC TCG AAT GTT CGC GAA GTT TCG-3′) described by Goldenberg *et al.*[19] was synthesized by Integrated DNA Technologies Incorporation (Coralville, IA). The probes were labeled with [γ-$^{32}$P]ATP using T4 polynucleotide kinase (Life Technologies, Rockfield, MD) and purified in push columns (Stratagene). For EMSA, 10 μg of nuclear proteins were preincubated with EMSA buffer (12 mmol/l HEPES pH 7.9, 4 mmol/l *Tris*-HCl pH 7.9, 25 mmol/l KCl, 5 mmol/l $MgCl_2$, 1 mmol/l EDTA, 1 mmol/l DTT, 50 ng/ml poly[d(I-C)], 12% glycerol (v/v), and 0.2 mmol/l PMSF) on ice for 10 min before addition of the radiolabeled oligonucleotide probe for an additional 10 min. Protein–nucleic acid complexes were resolved using a non-denaturing polyacrylamide gel consisting of 5% acrylamide (29:1 ratio of acrylamide/bisacrylamide) and run in 0.5 × TBE (45 mmol/l *Tris*-HCl, 45 mmol/l boric acid, 1 mmol/l EDTA) for 1 h at constant current (30 mA). Gels were transferred to Whatman 3 M paper, dried under a vacuum at 80°C for 1 h, and exposed to photographic film at −80°C.

### Statistical Analysis

All data are presented as mean ± standard deviation. Differences in mean concentrations between the four treatment groups were assessed by one-way analysis of variance (ANOVA). Pairwise analysis using Bonferroni tests were performed to determine whether the mean values of each group differed from each other. The differences were determined to be significant if the *P* value was found to be less than or equal to 0.05. Data were processed using Sigma Stat for Windows, Version 2.03 (SPSS Incorporation, Chicago, IL).

## RESULTS

### Concentration-response to LA

LPS stimulates an acute inflammatory response in RAW cells characterized by overproduction of nitric oxide. In fact, we detected over 120 μmol/l total nitrites and nitrates (NOx) in culture media in response to 100 ng/ml LPS (Fig. 1). LA inhibits nitric oxide synthesis in a dose dependent manner. Inhibition occurs at LA doses greater than 100 μmol/l. Interpolation of the curve indicates a half maximal response at approximately 500 μmol/l

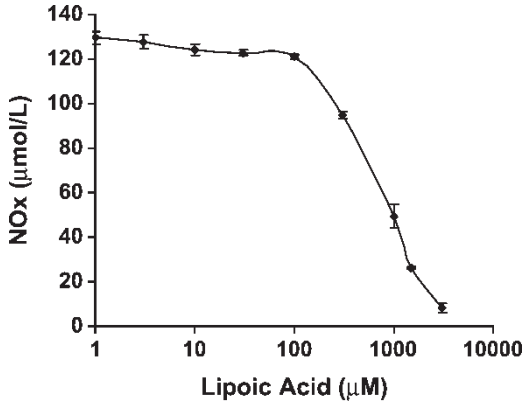


FIGURE 1 RAW 264.7 cells were treated with 100 ng/ml LPS for 18 h and the indicated concentrations of α-lipoic acid (LA). The *Y*-axis values represent the concentration of the sum of media nitrites and nitrates (NOx) derived from a modified Griess assay. The graph indicates a progressive decrease in nitric oxide synthesis with increasing concentrations of LA.

LA; therefore, this dose was used in all subsequent experiments. This dose has been used by others and is not cytotoxic to LPS-treated RAW cells.[15] We also tested doses of LA as high as 1000 μmol/l on LPS-treated RAW cells and did not observe cytotoxicity (not shown).

### iNOS Expression and Nitric Oxide Synthesis

Under control conditions RAW cells do not express iNOS protein (Fig. 2A); however, LPS induces a dramatic increase in this enzyme ($P < 0.001$). Treatment with LA or HS blunted the expression of iNOS to less than one-third and one-fourth the level, respectively, of iNOS expressed in cultures treated only with LPS ($P > 0.001$). The reduced expression of iNOS protein was consistent with reductions in total nitrates (NOx) in culture media (Fig. 2B). Culture media from unstimulated RAW cells contained little NOx. Treatment with LPS caused a profound increase in NOx, which was partially inhibited by pretreatment with LA ($P < 0.001$) or HS ($P < 0.001$).

### IκB-α

Others have reported that degradation of IκB-α starts within 4 min of 100 ng/ml LPS treatment in RAW cells and this effect is sustained for approximately 4 h.[20] We examined the effects of LPS on IκB-α degradation in whole cell homogenates 18 h after treatment. Immunoblotting analysis indicates that the level of IκB-α in cultures treated with LPS only, LPS plus LA, and LPS plus HS were 27, 70, and 30% of those in untreated cultures (Fig. 3) ($P < 0.001$, $P < 0.22$, $P < 0.001$, respectively). That the levels of IκB-α in the LA plus LPS group are only slightly lower than that in the control group indicates that,

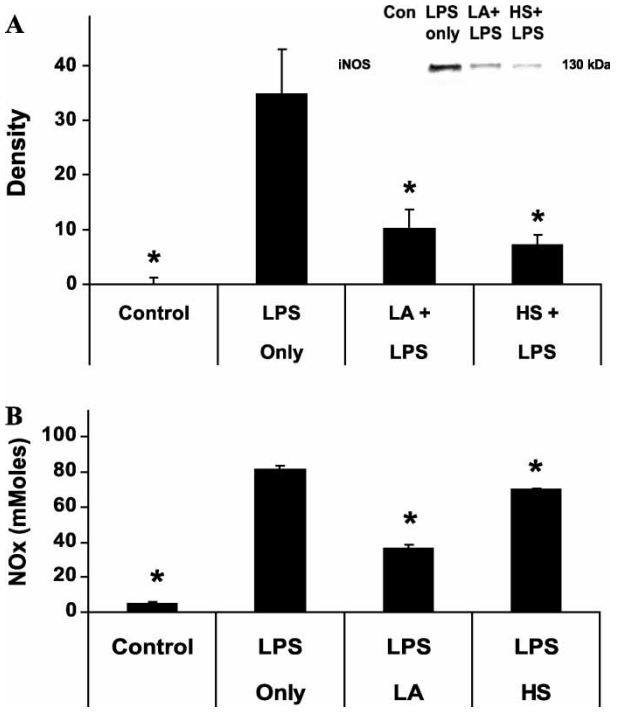


FIGURE 2 (A) Densitometry was performed on an immunoblot to quantify iNOS protein levels in protein extracts from four treatment groups (control, LPS only, LA pretreatment with LPS, and heat shock (HS) pretreatment with LPS). The analysis indicates that 18 h exposure to LPS induces expression of iNOS protein that is inhibited by treatment with LA or heat shocking (HS). No iNOS protein was detected in control cultures. The inset shows a representative band from each treatment group. An asterisk indicates a significant difference compared to values seen in the LPS only treatment group. (B) Bars represent measures of the sum of accumulated nitrites and nitrates (NOx) in culture media after an18 h exposure to LPS or control vehicle solution. The analysis indicates that LPS induces synthesis of nitric oxide, an effect that is inhibited by treatment with LA or heat shocking (HS). Very low levels of nitric oxide were detected in control cultures that were treated with the LPS vehicle solution.

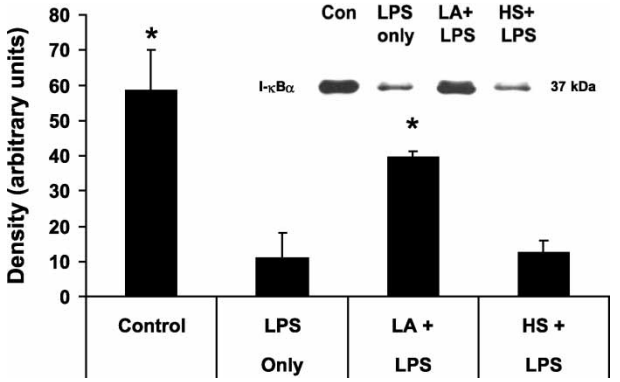


FIGURE 3 Western immunoblot analysis of I-κBα in whole cell protein extracts from control, LPS only, LA pretreatment with LPS, and HS pretreatment with LPS groups. Cultures were harvested 18 h after LPS treatment. Densitometry indicates that control cultures have high levels of I-κBα. LPS causes a dramatic reduction in I-κBα levels, indicative of phosphorylation and ubiquitination of this protein. LA, but not heat shocking, inhibits the disappearance of this 37 kDa protein. The inset shows a representative band from each treatment group. An asterisk indicates a significant difference compared to values seen in the LPS only treatment group.

unlike HS, LA is effective at reducing LPS-induced degradation of IkB-α 18 h after cell activation.

### NF-κB and p65

Electrophoretic mobility shift assay (Fig. 4A inset) confirms that LPS increases NF-κB nuclear translocation. Densitometric analysis (Fig. 4A) reveals that treatment with LPS for 45 min results in a 2-fold increase in the presence of NF-κB in the nuclear extracts ($56 \pm 43$ vs $113 \pm 20$ arbitrary units, $P < 0.05$). Treatment with LA or HS in the presence of LPS blunted the level of nuclear NF-κB ($38 \pm 5$ and $51 \pm 1$ arbitrary units; $P < 0.01$ and 0.03, respectively) levels which are similar to those found in control cultures ($P > 0.05$).

Under control conditions nuclear extracts from RAW cells express a low basal level of p65 protein (Fig. 4B). LPS treatment for 18 h results in a greater than 12-fold increase in p65 level in the nucleus ($5 \pm 3$ vs $67 \pm 0$ arbitrary units, $P < 0.001$). Treatment with LA or HS in the presence of LPS blunted the LPS-induced increase in p65 ($13 \pm 7$ and $38 \pm 8$ arbitrary units; $P < 0.001$ and 0.001, respectively). The nuclear level of p65 in LA treated cultures was similar to the baseline level of p65 measured in control cultures ($P > 0.05$). Although the nuclear level of p65 in LPS-activated heat shocked cultures was less than that measured in the LPS only group, the level of this protein was elevated above the baseline level of this protein measured in control cultures ($P < 0.001$).

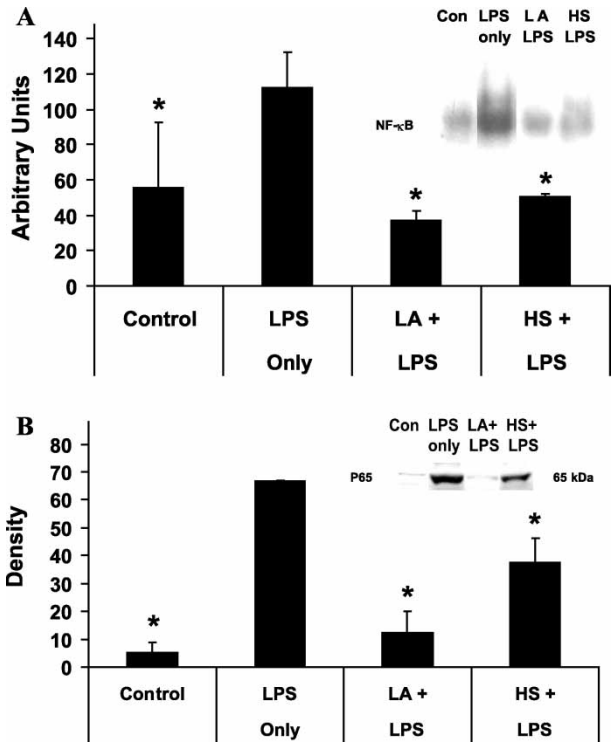


FIGURE 4 (A) Densitometry was performed following electrophoretic mobility shift assay to determine NF-κB binding activity of nuclear extracts from control, LPS only, LA pretreatment with LPS, and HS pretreatment with LPS groups. All cultures were harvested after 45 min of LPS exposure. The analysis indicates that LPS causes activation and translocation of NF-κB, processes which are inhibited by LA and HS pretreatment. An asterisk indicates a significant difference compared to values seen in the LPS only treatment group. Inset shows a representative lane for the four treatment groups from the gel mobility shift assay. Treatment order is the same as that listed on the *x*-axis label. (B) Western immunoblot analysis of nuclear protein extracts from RAW 264.7 cells using an antibody to p65. All cultures were harvested after 45 min of LPS exposure. LPS causes an increase in the level of this NF-κB protein subunit in the nucleus. LA and HS pretreatment inhibit the LPS-induced increase in the nuclear levels of p65. These results are consistent with results from the gel shift assay (Fig. 3).

### HSP-70 and HSF-1

Immunoblotting analysis was performed to determine whether the anti-inflammatory properties of LA are mediated by an induction of HSP-70 protein expression. Heat shocking, a known inducer of the HSP-70, was used as a positive control. Our data confirm that heat shock, in the presence or absence of LPS, induces similarly high levels of HSP-70 protein in RAW cells ($125 \pm 14$ vs $121 \pm 9$ arbitrary units, $P > 0.05$) (Fig. 5). Untreated control cultures express baseline levels of HSP-70 that are no different from cultures treated with LA alone ($6 \pm 6$ vs $10 \pm 7$ arbitrary units, $P > 0.05$), indicating that LA by itself does not induce expression of this protein. The level of HSP-70 in LA plus LPS treated cultures is increased compared to that in untreated control cultures ($45 \pm 19$ vs $6 \pm 7$ arbitrary units, $P < 0.05$). The absence of a difference in HSP-70 levels between LA plus LPS and LPS only cultures

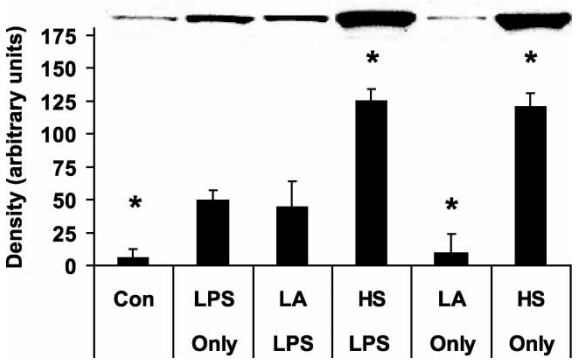


FIGURE 5 Western immunoblot analysis of HSP-70 in whole cell protein extracts from control, LPS only, LA pretreatment with LPS, HS pretreatment with LPS groups, LA only and HS only groups. Representative 70 kDa HSP-70 protein bands from each treatment group are shown directly above the respective treatment groups. Cultures were harvested 18 h after LPS treatment. Densitometry indicates that treating cells with LA does not change HSP-70 protein levels compared to the untreated control cultures. HSP-70 levels are elevated in all the groups treated with LPS compared to the untreated control group. Among the groups treated with LPS, heat shock, but not LA causes an elevation in HSP-70. An asterisk indicates a significant difference ($P < 0.05$) from the LPS only treatment group.

($45 \pm 19$ vs $50 \pm 10$ arbitrary units, $P > 0.05$) indicates that the increase in HSP-70 observed in the LA plus LPS cultures is due specifically to LPS. Thus, there is no evidence that the inhibition of LPS stimulated iNOS protein expression, NO synthesis, NF-κB activation, p65 protein expression, and IκB-α degradation by LA is mediated by HSP-70. Given no evidence that LA is mediated by a heat shock response and that LA does not affect an LPS-induced heat shock response, we further examined the possibility that LA inhibits a heat shock-induced heat shock response. In a separate experiment we pretreated cells with 0.5 mM LA for 2 h, heat shocked them for 1 h, and harvested the cells 18 h later. Another group of cells was treated similarly except that they received vehicle solution instead of LA. HSP-70 levels in these two groups, measured by Western analysis were no different ($79 \pm 7$ vs $76 \pm 3$ arbitrary units, $P > 0.05$). These data indicate that LA does not inhibit a heat shock-induced heat shock response.

RAW cells express a high baseline level of nuclear HSF-1 (Fig. 5B). All three groups treated with LPS for 45 min exhibit slightly but significantly higher levels of HSF-1. Furthermore, the level of HSF-1 in the HS group is significantly elevated above that of the LPS only group ($P < 0.01$) and the LA plus LPS group ($P < 0.05$).

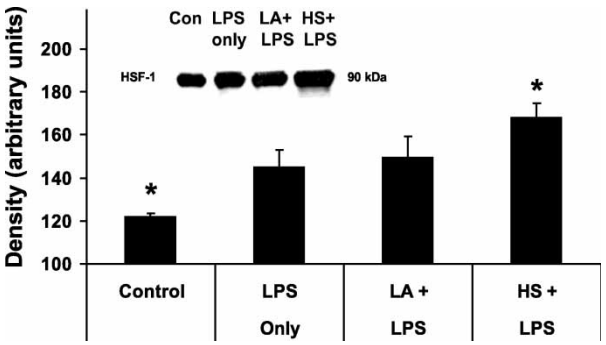


FIGURE 6 Western immunoblot analysis of HSF-1 in nuclear extracts from control, LPS only, LA pretreatment with LPS, and HS pretreatment with LPS groups. Cultures were harvested 45 min after LPS treatment. An increase in the nuclear levels of HSF-1 relative to control cultures is seen in cultures treated with LPS only or those pretreated with LA and then exposed to LPS. Heat shocking prior to LPS treatment results in a slightly greater elevation in HSF-1 compared to the two other groups treated with LPS.

## DISCUSSION

Heat shock protein-70 (HSP-70) and HSF-1 are key mediators of the heat shock response. We reasoned that elevated levels of nuclear HSF-1 and whole cell HSP-70, above that induced by LPS, would be evidence of a heat shock response. Indeed, prior exposure of cells to heat stress results in the highest levels of whole cell HSP-70 and nuclear HSF-1 compared to other treatment groups. Lipoic acid does not induce the expression of HSP-70 above the unstimulated baseline control level or HSF-1 above that caused by LPS alone. This evidence supports the idea that LA suppresses inflammation independent of heat shock protein activation.

Administration of LPS alone increased the levels of nuclear HSF-1 and HSP-70 in RAW cells (Figs. 5 and 6). LPS has been shown to induce HSP-70 expression in the heart, liver and lungs of rats.[21,22] We have observed that the same concentration of LPS causes induction of heme oxygenase-1 (HSP-32) in RAW cells (unpublished observation). Induction of heme oxygenase by LPS is indicative of oxidative stress.[23,24] LA suppressed the induction of heme oxygenase-1 in a rat model of oxidative stress.[25] We found no effect of LA on LPS or heat shock induced expression of HSP-70, suggesting that LA cannot regulate the heat shock response induced by either LPS or heat shocking.

In the present study, we demonstrate that LA and HS pretreatment inhibit the expression of iNOS and NO synthesis in mouse macrophages (RAW 264.7 cells) activated with endotoxin. The inhibition of IκB-α degradation and NF-κB activation associated with LA pretreatment of endotoxin-treated RAW cells suggests that the inhibitory effects on iNOS expression and NO synthesis are mediated, in part, through the NF-κB signaling pathway. Indeed, previous work using RAW 264.7 cells has identified NF-κB enhancer elements in the iNOS promoter that permit iNOS induction by cytokines and LPS.[26] Finally, because our data show that heat shock, but not LA, increases expression of HSF-1 and HSP-70, we conclude that the inhibition of the NF-κB signaling pathway by LA is independent of induction of a heat shock response.

Results of this study are consistent with other reports showing that LA inhibits endotoxin-induced nitrite accumulation in RAW 264.7 cells by inhibition of translocation of NF-κB into the nucleus.[15] We extend those findings by demonstrating an inhibitory effect of LA on degradation of the NF-κB inhibitory protein, I-κBα, and subsequent iNOS protein expression. We observed high levels of IκB-α in LA pretreated RAW cells exposed to LPS for 18 h. This finding demonstrates that LA suppresses LPS-induced degradation of I-κBα and likely inhibits the NF-κB signaling for up to 18 h.

Shanley *et al.*[27] demonstrated complete degradation of I-κBα in RAW 264.7 cells within 15 min of LPS treatment and that I-κBα levels return to normal within the next 15 min. When cells were subjected to heat shocking for 45 min at 43°C, LPS-mediated degradation of IκB-α was completely blocked. We observed low levels of IκB-α in the cultures

exposed to LPS alone for 18 h, indicating continued I-κBα degradation due to LPS. We also observed that heat shocking afforded no stabilization of the IκB-α 18 h after LPS treatment. Thus, our data suggest that recovery from LPS-induced IκB-α degradation in RAW cells observed by Shanley *et al.*[27] is likely to be transient or model specific. Our data also suggest that the stabilization of I-κBα caused by heat shocking is either transient or model specific.

Relative to changes in iNOS expression, heat shock caused a disproportionate change in nitrates and nitrites in the culture media. Heat shock protein-90 has been shown to enhance iNOS activity[28] and increased expression of this heat shock protein may explain our finding that heat shock suppresses iNOS expression more than nitrate and nitrite accumulation in culture media.

Pyrrolidine dithiocarbamate (PDTC), a low molecular weight thiol compound with antioxidant properties, is one of few therapies that activates the heat shock response and inhibits inflammatory signaling.[29] We have observed a 10-fold increase in the level of HSP-70 in LPS-treated RAW 264.7 cells pretreated with PDTC (not shown), indicating this antioxidant is a potent inducer of a heat shock response. The activation of HSF-1 and subsequent expression of HSP-70 by PDTC is suggested to be due to its activities as a pro-oxidant and thiol modulator rather than antioxidant.[30] PDTC causes an increase in oxidized GSH and induces activation of HSF-1 in rat fibroblasts, effects that can be blocked by treatment with an antioxidant, such as, *n*-acetylcysteine. Cell culture studies indicate that PDTC can cause inhibition or activation of NF-κB, depending upon the type of cell studied, and raise concern that the differential effects may have implications for studies of PDTC as an immunomodulatory drug.[31] We have observed cytotoxicity with PDTC in LPS-treated RAW 264.7 cells (not shown), but not with LA (Fig. 1). Others have shown dose-dependent neurotoxicity in rats with PDTC.[32] On the other hand, LA has a proven clinical track record of safety for treatment of symptoms of diabetic polyneuropathy.[33,34]

One limitation to this study relates to the use of 0.5 mM LA. Although this concentration is an effective inhibitor of iNOS, NO synthesis, IκB-α degradation, and NF-κB activation in RAW 264.7 cells, it is 100-fold higher than the plasma concentration of LA (1.1 μg/ml or 5 μM) measured in normal healthy male volunteers after an oral administration of 1 g of LA.[11] On the other hand, pretreating adult rats with 0.5 mM intravenous LA reduces ischemia-reperfusion injury in the liver.[35]

In summary, these studies demonstrate that LA inhibits LPS-induced iNOS expression and activity in RAW 264.7 cells by inhibition of translocation of NF-κB into the nucleus, an effect that is not meditated by a heat shock response. The hypothesis that the cytoprotective effects of LA are due to triggering of the heat shock response[17] cannot be supported by these experiments conducted with mouse macrophages. Additionally, these results provide rationale to further examine the anti-inflammatory effects of LA in an animal model of endotoxemia. We would predict that such studies would demonstrate that LA reduces LPS-induced inflammation by inhibiting NF-κB activation and the NF-κB-dependent expression of several pro-inflammatory proteins.

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