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Original Research Communication

Heat Shock and 5-Azacytidine Inhibit Nitric Oxide Synthesis and Tumor Necrosis Factor-α Secretion in Activated Macrophages

HAN DO KIM,[1] HO SUNG KANG,[1] GERALD RIMBACH,[2] and YOUNG CHUL PARK[1,2]

ABSTRACT

To elucidate the role of stress response during macrophage activation, the effects of heat shock and the amino acid analog, 5-azacytidine on nitric oxide (NO) production, tumor necrosis factor-alpha (TNF-α) secretion, and heat shock protein (HSP) synthesis have been studied in murine peritoneal macrophages (C57BL/6). Heat shock (1 hr at 43°C) or 5-azacytidine markedly inhibited the release of NO into the medium from interferon-γ (IFN-γ) plus lipopolysaccharide (LPS)-stimulated macrophages. Although heat shock significantly decreased TNF-α secretion only at the initiation stage of macrophage stimulation, 5-azacytidine treatment resulted in a more prolonged reduction in the secretion of TNF-α. When heat-shocked cells were stimulated with IFN-γ plus LPS under normal culture conditions at 37°C, the heat shock-induced inhibition of NO release reversed progressively with increasing recovery time. Although the total amount of cellular HSP72 measured by Western blot increased time-dependently over 7 hr, newly synthesized HSP72 measured by [^{35}S]methionine incorporation was evident only after 1 and 3 hr of recovery time after heat shock treatment. At these time points, the lowest nitrite accumulation and TNF-α secretion into the medium was evident. It is concluded that signaling pathways related to newly synthesized HSP such as HSP72 are implicated in the down regulation of NO synthesis and TNF-α secretion in macrophages. Antiox. Redox Signal. 1, 297–304.

INTRODUCTION

MACROPHAGES PERFORM SPECIFIC IMMUNOLOGIC and nonspecific inflammatory functions (Unanue and Allen, 1987). These functions of macrophages may be mediated by reactive nitrogen species (Stuehr and Nathan, 1989), reactive oxygen species (ROS) (Fischer and Bostick-Bruton, 1982), or tumor necrosis factor-α (TNF-α) (Larrick and Wright, 1990). Activated macrophages can generate large amounts of nitric oxide (NO) from L-arginine by the action of inducible NO synthase (iNOS). NO is an important intracellular and intercellular regulatory molecule of multiple biological functions, including macrophage-mediated cytotoxicity, neurotransmission, and smooth-muscle relaxation (Moncada *et al.*, 1991; Yui *et al.*, 1991; Lowenstein and Snyder, 1992). The activity of iNOS in macrophage is regulated by mediators such as interleukin-2 (IL-2), interferon-γ (IFN-γ), and inflammatory stimuli such as bacterial lipopolysaccharide (LPS) (Stuehr and Marletta, 1987; Narumi *et al.*, 1990). It has

[1]Department of Molecular Biology, Pusan National University, Pusan 609-735, Korea.
[2]Department of Molecular and Cell Biology, University of California, Berkeley, California 94720-3200.

also been shown that the production of TNF-α is crucial for the synergistic induction of NO synthesis in IFN-γ and/or LPS-stimulated murine macrophages (Green *et al.*, 1990; Jun *et al.*, 1995).

Stress response is a primitive defense program that is induced in cells exposed to toxic insults such as heat, amino acid analogs, heavy metals, and ischemia-reperfusion. Induction of the stress response leads to the synthesis of various heat shock proteins (HSP). Because the HSP are highly conserved through evolution, it has been assumed that HSP serve some universally important functions. In particular, HSP of the 70-kDa family such as HSP72/73 not only confers thermotolerance but also protects against ultraviolet light-induced injury, oxygen radical toxicity, myocardial ischemia, *Escherichia coli* sepsis, and acute lung injury. Moreover, recent studies suggest that HSP participate in crucial phenomena such as protein import and assembly, protection from environmental stress, immunity, autoimmunity, and cancer (Ayala *et al.*, 1992; Jäättelä and Wissing, 1992).

Despite considerable data describing the biological activities of NO, the molecular mechanisms of macrophage activation involving NO and TNF-α remain unknown. Therefore, extensive research on the interaction and cellular regulation of NO and TNF-α synthesis is necessary to develop an understanding of the various processes that regulate inflammation. In this study, the effect of stress response on NO synthesis was investigated in murine peritoneal macrophages stimulated with IFN-γ plus LPS. We also tested whether this stress response is associated with an inhibitory effect on the production of TNF-α.

MATERIALS AND METHODS

Materials

Thioglycollate (TG) broth (Brewer) was purchased from DIFCO (Detroit, MI). Murine rTNF-α (1×10^6 U/ml), rabbit anti-murine TNF-α polyclonal antibody (neutralizing), and hamster anti-murine TNF-α monoclonal antibody were purchased from Genzyme (München, Germany). Anti-murine iNOS and anti-HSP antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). LPS (phenol extracted *Salmonella enteritidis*), 5-azacytidine (5-Aza), sodium nitrite, sulfanilamide, *N*-(1-naphthyl)-ethylenediamine dihydrochloride, protease inhibitor cocktail, Tween-20, bovine serum albumin (BSA), phosphatase-conjugated goat anti-rabbit immunoglobulin G (IgG), and *p*-nitrophenyl phosphate were purchased from Sigma Chemical Co. (St. Louis, MO). RPMI containing L-arginine (200 mg/L), fetal calf serum (FCS), Hanks' balanced salt solution (HBSS), and other tissue culture reagents were purchased from Life Technologies (Gaithersburg, MD).

Macrophage culture and metabolic labeling

The original stock of C57BL/6 mice was purchased from The Dae Han Animal Center (Seoul, Korea) and the mice were maintained in the Department of Molecular Biology, Pusan National University. TG-elicited macrophages were harvested 3 days after intraperitoneal (i.p.) injection of 2.5 ml of TG into mice and isolated as reported previously (Narumi *et al.*, 1990). Then, cells were suspended in RPMI 1640, which was supplemented with 10% (vol/vol) FCS, in either 24-well tissue culture plates (5×10^5 cells/well) or 60-mm-diameter plastic petri dishes (5×10^6 cells/well), incubated for 3 hr at 37°C in an atmosphere of 5% CO_2. Nonadherent cells were removed by suction, and then freshly prepared complete medium was added with the indicated experimental reagents. To induce the heat shock (HS) response, cells were incubated for 1 hr at 43°C and then recovered to normal growth temperature (37°C) for up to 1 hr. For [^{35}S]methionine labeling, cells were washed in methionine-free RPMI three times and labeled in methionine-free RPMI plus [^{35}S]methionine supplemented with 2% dialyzed FCS for the last 1 hr of recovery time.

Nitrite concentration

NO secretion in cultured macrophages was measured by a microplate assay method, as described earlier (Green *et al.*, 1982). To measure nitrite (NO_2^-), 100 μL of the supernatants in cultured macrophages were collected and

mixed with an equal volume of the Griess reagent (1% sulfanilamide, 0.1% *N*-(1-naphthyl)-ethylenediamine dihydrochloride, 2.5% H_3PO_4) and incubated for 10 min at room temperature. Nitrite concentration was determined by measuring the absorbance at 540 nm in a Titertek Multiskan spectrophotometer (Flow Laboratories, North Ryde, Australia). $NaNO_2$ was used for external calibration. Cell-free medium alone contained 5–8 μM of nitrite; this value was determined in each experiment and subtracted from the value obtained for the cells.

Western blot analysis of iNOS and HSP

The cell lysates were separated electrophoretically using 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) as described by Laemmli (1970), and then the gel was transferred to 0.45 μM nitrocellulose paper. The blot was blocked with 5% nonfat milk in phosphate-buffered saline (PBS) containing 0.1% Tween-20 (PBS-T) for 1 hr at room temperature, washed, and incubated with anti-iNOS and anti-HSP antibodies (HSP72, HSP73) for 1 hr (Towbin *et al.*, 1979). After washing in PBS-T four times, the blot was developed using horseradish peroxidase-conjugated secondary antibody. After washing four times, bands corresponding to iNOS and HSP were visualized using enhanced chemiluminescence (ECL) detection system according to the recommended procedure (Amersham).

TNF-α secretion

TNF-α secretion was measured by modification of an enzyme-linked immunosorbent assay (ELISA), as described earlier (Scuderi *et al.*, 1986). For the ELISA, 96-well plates were coated with 6.25 ng/well of murine monoclonal antibody with specificity for murine TNF-α. Before use and between subsequent steps in the assay, coated plates were washed twice with PBS containing 0.05% Tween-20 and twice with PBS alone. For the standard curve, rTNF-α was added to serum previously determined to be negative for endogenous TNF-α. After exposure to medium, assay plates were sequentially exposed to rabbit anti-TNF-α, phosphatase-conjugated goat anti-rabbit IgG, and *p*-nitrophenyl phosphate. Optical density readings at 410 nm were taken using a Emax 96-well microtest plate spectrophotometer (Molecular Devices, Menlo Park, CA).

RESULTS

To assess the role of stress response in macrophage activation, first the effects of HS or the amino acid analog 5-Aza on NO synthesis in murine peritoneal macrophages

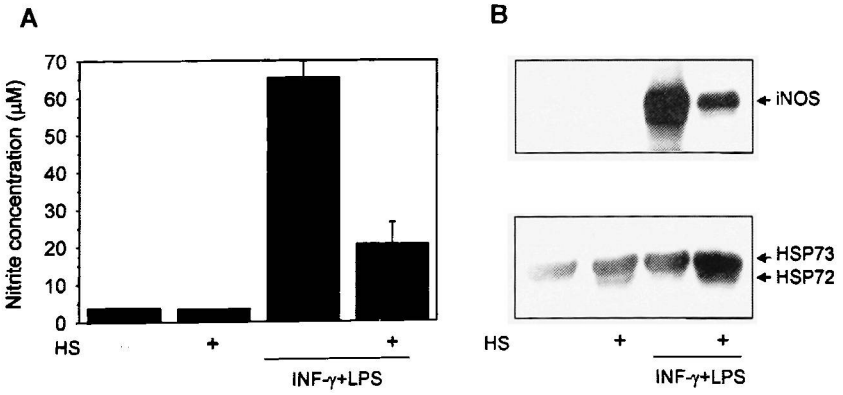

FIG. 1. Effect of HS on the synthesis of NO by IFN-γ plus LPS-stimulated murine peritoneal macrophages. A. TG-elicited macrophages (5×10^5) were incubated at 43°C for 1 hr, and recovered at 37°C for 1 hr. Then, the cells were stimulated with IFN-γ (5 U/ml) plus LPS (10 ng/ml), and incubated for another 24 hr. The amount of NO released by macrophages was measured by the method of Griess (nitrite). Results are presented as the means ±SD of five independent cell preparations. **B.** Macrophages (5×10^6 cells/60-mm diameter petri dishes) were incubated as described in **A**. Then, the cell lysates were analyzed by SDS-PAGE, and iNOS and HSP were detected by Western blot analysis.

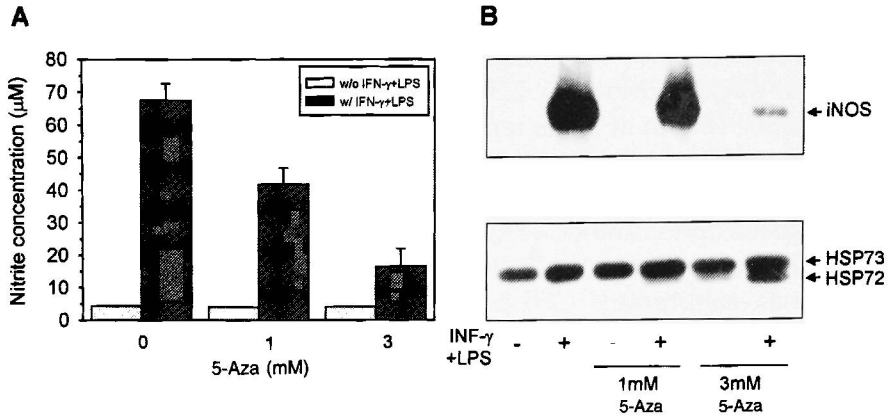

FIG. 2. Effect of 5-Aza on the synthesis of NO by IFN-γ plus LPS-stimulated macrophages. **A**. TG-elicited macrophages (5×10^5) were incubated for 1 hr in the presence or absence of various concentrations of 5-Aza. Then, the cells were stimulated with IFN-γ (5 U/ml) plus LPS (10 ng/ml), and incubated for another 24 hr. The amount of NO released by macrophages was measured by the method of Griess (nitrite). Results are presented as the means ±SD of five independent cell preparations. **B**. Macrophages (5×10^6 cells/60-mm diameter petri dishes) were incubated as described in **A**. Then, the cell lysates were analyzed by SDS-PAGE, and iNOS and HSP were detected by Western blot analysis.

(C57BL/6) were investigated. TG-elicited macrophages were exposed to HS (1 hr at 43°C) or 5-Aza to induce the stress response followed by the treatment of IFN-γ plus LPS. The amount of NO released was measured as nitrite by the method of Griess. Stimulation of the cells with IFN-γ plus LPS resulted in an increased accumulation of nitrite in the medium. However, nontreated control cells produced negligible amounts of NO (<5 μ*M*). As shown in Fig. 1A, HS inhibited the production of NO released by stimulated macrophages. To determine whether the decreased nitrite accumulation was correlated with changes in iNOS expression, the amount of iNOS was analyzed at the protein level by Western blotting (Fig. 1B). In unstimulated macrophages, iNOS was not detectable. Similarly, when macrophages were preincubated in medium containing 5-Aza for 1 hr, and then stimulated with IFN-γ plus LPS for 24 hr, the amount of NO secreted into culture supernatant was significantly reduced in a dose-dependent manner (Fig. 2A,B).

TNF-α secretion, which can be induced by IFN-γ and/or LPS, is known to be crucial for the synergistic induction of NO synthesis in murine peritoneal macrophages. To examine whether stress response evoked a reduction of NO synthesis by regulation of the TNF-α production, the amount of TNF-α secreted from macrophages pretreated with HS or 5-Aza was assessed quantitatively. Although the amount of NO released was decreased both by HS or 5-Aza, present data demonstrate a discrepancy in TNF-α secretion from macrophages after treatment with HS or 5-Aza. After 24 hr of stimulation the level of TNF-α secretion in the culture medium was decreased by 5-Aza but no effect of HS on TNF-α secretion at this time-

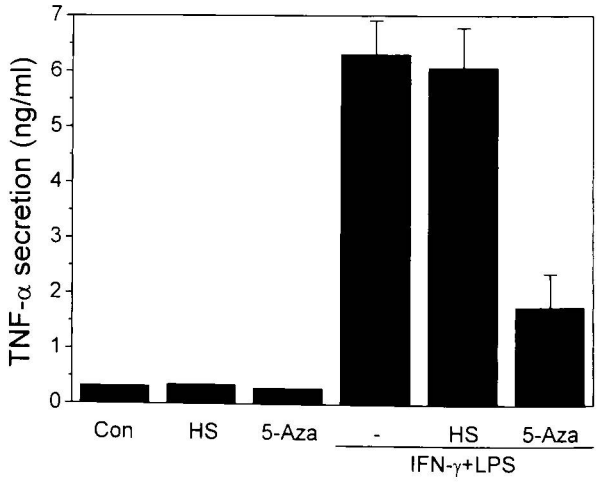

FIG. 3. Effects of HS or 5-Aza on the secretion of TNF-α by rIFN-γ plus LPS-stimulated macrophages. TG-elicited macrophages (5×10^5) were pretreated with HS or 5-Aza (3 m*M*) as described in Figs. 1 and 2. Then, the cells were stimulated with IFN-γ (5 U/ml) plus LPS (10 ng/ml). After 24 hr of stimulation, supernatants were collected, and the amount of TNF-α secreted by macrophages was determined by specific ELISA. Values are means ±SD of three experiments.

point was evident (Fig. 3). However, HS markedly decreased TNF-α secretion at the initiation stage of macrophage stimulation (Fig. 4). In addition, to determine the role of recovery time in the HS-induced inhibition of NO, cells were stimulated with IFN-γ plus LPS after various time points (1, 3, 5, or 7 hr) of recovery under normal culture conditions of 37°C. Interestingly, it was observed that the HS-induced inhibition of NO release was progressively reversed with increasing recovery time (Fig. 5). The possibility that the inhibition of NO release by HS was related to the synthesis or expression of HSP was then investigated. Macrophages were labeled with [^{35}S]methionine for the last 1 hr of recovery time. Although the total amount of existing cellular HSP72 measured by Western blot increased at later stages of recovery, newly synthesized HSP72 was only evident after 1 and 3 hr of recovery after HS treatment (Fig. 6A,B).

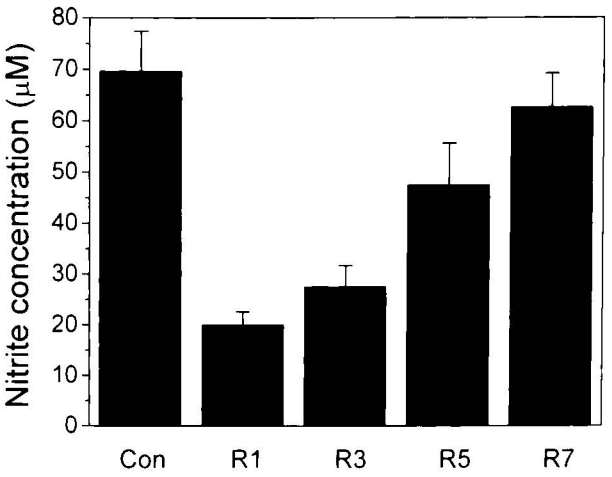

FIG. 5. Reversibility of the HS-induced reduction of NO release from IFN-γ plus LPS-stimulated macrophages. TG-elicited macrophages (5×10^5) were incubated at 43°C for 1 hr and allowed to recover at 37°C for 1, 3, 5, or 7 hr. Then, cells were stimulated with IFN-γ (5 U/ml) plus LPS (10 ng/ml), and incubated for another 24 hr. The amount of NO released by macrophages was measured by the method of Griess (nitrite). Results are presented as the means ±SD of three experiments.

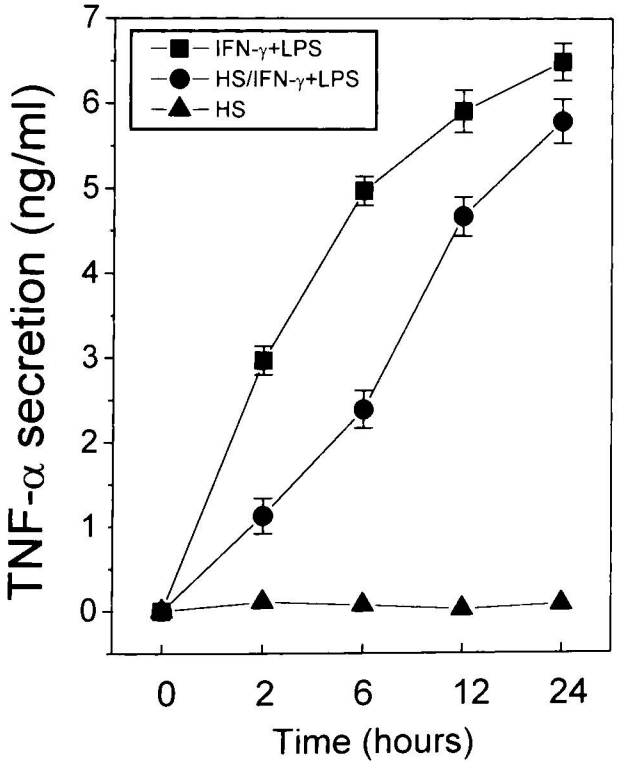

FIG. 4. Effect of HS on rIFN-γ plus LPS-induced TNF-α secretion at the early stage of stimulation. TG-elicited macrophages (5×10^5) were incubated at 43°C for 1 hr, and recovered at 37°C for 1 hr. Then, the cells were stimulated with IFN-γ (5 U/ml) plus LPS (10 ng/ml). At the indicated time points, supernatants were collected and assayed for TNF-α with an ELISA. Values are means ±SD of three experiments.

DISCUSSION

In macrophages, NO is a cytotoxic mediator and contributes to the antimicrobial and tumoricidal activity of these cells (Stuehr and

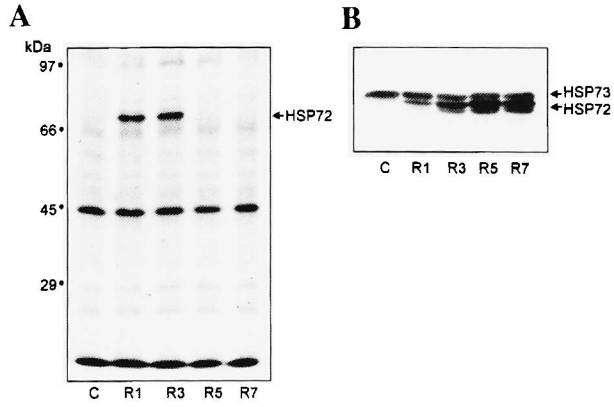

FIG. 6. Synthesis and expression of HSP during recovery time. A. TG-elicited macrophages (2×10^6 cells/33-mm diameter petri dishes) were incubated at 43°C for 1 hr and allowed to recover at 37°C for 1, 3, 5, or 7 hr. Then, the cells were labeled with [^{35}S]methionine for the last 1 hr and the proteins were analyzed by SDS-PAGE and fluorography. **B.** Macrophages (5×10^6 cells/60-mm diameter petri dishes) were incubated as described in **A**. After recovery time, the cell lysates were analyzed by SDS-PAGE, and HSP were detected by Western blot analysis.

Nathan., 1989; Nathan and Hibbs, 1991; Yui *et al.*, 1991). However, high NO production has been associated with oxidative stress and with the pathophysiology of various diseases such as arthritis, diabetes, septic shock, and autoimmune and chronic inflammation (Liu *et al.*, 1993; Bo *et al.*, 1994; Sakurai *et al.*, 1995). The induction of iNOS gene expression is tightly regulated by mediators such as IL-2, IFN-γ, and LPS. Furthermore, it is well documented that the proinflammatory cytokine TNF-α is crucial for the synergistic induction of NO synthesis in IFN-γ and LPS-stimulated macrophages (Hirvonen *et al.*, 1996). Cells exposed to various environmental stresses such as heat, amino acid analogs, heavy metals, and ultraviolet light respond by synthesizing a unique set of polypeptides termed heat shock or stress proteins. The heat shock response often includes rapid expression of HSP, which may protect cells either by facilitating the renaturation of partially denaturated proteins or by restricting inflammatory responses themselves. In addition, overexpression of HSP70 has been shown to protect tumor cells against cytokine cytotoxicity (Jäättelä, 1993; Jacquier-Sarlin *et al.*, 1994). However, the underlying mechanisms by which HSP modulate NO synthesis and TNF-α secretion are still unclear. Therefore, in the present study, the effect of stress response on HSP, NO, and TNF-α synthesis as well as its interrelationship in IFN-γ plus LPS-stimulated macrophages was investigated.

Present results clearly demonstrate that 5-Aza and HS markedly inhibited the IFN-γ plus LPS-induced NO production in macrophages and possibly thereby decreased TNF-α secretion via an autoregulatory loop (Lis and Wu, 1993). In comparison to HS, the cytidine analog 5-Aza, known to be capable of altering the expression of certain genes, resulted in a longer-lasting decrease in TNF-α secretion, which was still evident after 24 hr (Andrews *et al.*, 1989). A possible explanation for the different kinetics in the inhibition of the secretion of TNF-α between HS and 5-Aza-treated cells may be provided by differences in the duration of the stress exposure. Whereas HS treatment lasted only for 1 hr, cells were continuously exposed to the amino acid analog 5-Aza. The observed decrease in NO production and TNF-α secretion in the 5-Aza or HS-treated macrophages seems to be an important cellular response that might reflect the attempt of the cell to protect itself from deleterious environmental stimuli (Maulik *et al.*, 1994). Moreover, recent data have shown that HSP may protect from TNF-α toxicity by inhibiting the action of reactive oxygen species on mitochondrial membrane potential and DNA damage (Cossarizza *et al.*, 1995).

Similar to our findings in macrophages, it has been also reported in brain glial cells that HS reduced NO synthesis by impairing cytosolic iNOS activity, steady-state mRNA levels, and gene promotor activity (Feinstein *et al.*, 1996). It should be considered that the presence of a consensus sequence in the promoter region of iNOS for the binding of nuclear factor kappa B (NF-κB) and the inhibition of iNOS expression with the inhibition of NF-κB establishes an essential role of NF-κB activation in the induction of iNOS. Because HS reduced LPS-induced nuclear accumulation of the transcription factor NF-κB p65 subunit, it is suggested that the perturbation of NF-κB activation suppress iNOS expression, thereby limiting destructive inflammation (Baeuerle and Henkel, 1994). Furthermore, it known that different proinflammatory cytokines such as TNF-α bind to their respective receptors and induce iNOS gene expression via activation of NF-κB (Kleinert *et al.*, 1996). Under the conditions investigated, HS resulted in a significant decrease in TNF-α secretion at the initiation stage of macrophage activation. Therefore, it is possible that the impaired TNF-α secretion under HS conditions might lead to an inhibition of NF-κB activation, thereby subsequently down regulating iNOS gene expression, which might be one possible factor for the observed decrease in iNOS protein levels and nitrite accumulation in the present study. It is also hypothesized that HSP of the 70-kDa family can enter the nucleus, after which a simple competition for nuclear pore complexes may occur. Although the precise molecular mechanisms are not known, it is alternatively postulated by Feinstein and coworkers (1996) that HSP could impede NF-κB activation by a direct interaction with ankyrin domains present in IκB.

As described previously (Snyder *et al.*, 1992),

in HS-treated macrophages newly synthesised HSP72 measured by [^{35}S]methionine incorporation was apparent only after 1 and 3 hr of recovery time and was no longer apparent at later stages after thermal shock. Interestingly, during the first 1–3 hr after HS treatment, the lowest nitrite accumulation and TNF-α secretion into the medium were evident, suggesting that signaling pathways related to newly synthesized HSP such as HSP72 are important determinants of the down regulation of NO synthesis and TNF-α secretion. However, the exact nature of the primary signal that regulates the heat shock response in macrophages remains to be clarified.

The results of this study clearly demonstrate that stress response can significantly block NO synthesis and TNF-α secretion. This might be an important cellular mechanism by which macrophages defend themselves from further damage. Signaling pathways related to the *de novo* synthesis of HSP appear to have a key role in regulating NO and TNF-α secretion in activated macrophages.

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ABBREVIATIONS

5-Aza, 5-azacytidine; BSA, bovine serum albumin; ECL, enhanced chemiluminescence; ELISA, enzyme-linked immunosorbent assay; FCS, fetal calf serum; HBSS, Hanks' balanced salt solution; HS, heat shock; HSP, heat shock protein; IFN-γ, interferon-γ; IgG, immunoglobulin G; IL-2, interleukin-2; LPS, lipopolysaccharide; NF-κB, nuclear factor kappa B; NO, nitric oxide; iNOS, inducible NO synthase; PBS, phosphate-buffered saline; ROS, reactive oxygen species; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; TNF-α, tumor necrosis factor-alpha; TG, thioglycollate.

REFERENCES

ANDREWS, D.F., NEMUNAITIS, J., TOMPKINS, C, and SINGER, J.W. (1989). Effect of 5-azacytidine on gene expression in marrow stromal cells. Mol. Cell. Biol. **9,** 2748–2751

AYALA, A., KISALA, J.M., FELT, J.A., PERRIN, M.M., and CHAUDRY, I.H. (1992). Does endotoxin tolerance prevent the release of inflammatory monokines (interleukin 1, interleukin 6, or tumor necrosis factor) during sepsis? Arch. Surg. **127,** 191–197.

BAEUERLE, P.A., and HENKEL, T. (1994). Function and activation of NFkappa B in the immune system. Annu. Rev. Immunol. **12,** 141–179.

BO, L., DAWSON, T.M., WESSELINGH, S., MORK, S., CHOI, S., KONG, P.A., HANLEY, D., and TRAPP, B.D. (1994). Induction of nitric oxide synthase in demyelinating regions of multiple sclerosis brains. Ann. Neurol. **36,** 778–786.

COSSARIZZA, A., COOPER, E.L., QUAGLINO, D., SALVIOLI, S., KALACHNIKOVA, G., and FRANCESCHI, C. (1995). Mitochondrial mass and membrane potential in coelomocytes from the earthworm Eisenia foetida: studies with fluorescent probes in single intact cells. Biochem. Biophys. Res. Commun. **214,** 503–510.

FEINSTEIN, D.L., GALEA, E., AQUINO, D.A., LI, G.C., XU, H., and REIS, D.J. (1996). Heat shock protein 70 suppresses astroglial-inducible nitric-oxide synthase expression by decreasing NFkappaB activation. J. Biol. Chem. **271,** 17724–17732.

FISHER, R.I., and BOSTICK-BRUTON, F. (1982). Depressed T cell proliferative responses in Hodgkin s disease: role of monocyte-mediated suppression via prostaglandins and hydrogen peroxide. J. Immunol. **129,** 1770–1774.

GREEN, L.C., WAGNER, D.A., GLOGOWSKI, J., SKIPPER, P.L., WISHNOK, J.S., and TANNENBAUM, S.R. (1982). Analysis of nitrate, nitrite, and [15N]nitrate in biological fluids. Anal. Biochem. **126,** 131–138.

GREEN, S.J., CRAWFORD, R.M., HOCKMEYER, J.T., MELTZER, M.S., and NACY, C.A. (1990). Leishmania major amastigotes initiate the L-arginine-dependent killing mechanism in IFN-gamma-stimulated macrophages by induction of tumor necrosis factor-alpha. J. Immunol. **145,** 4290–4297.

HIRVONEN, M.R., BRUNE, B., and LAPETINA, E.G. (1996). Heat shock proteins and macrophage resistance to the toxic effects of nitric oxide. Biochem. J. **315,** 845–849.

JÄÄTTELÄ, M. (1993). Overexpression of major heat shock protein hsp70 inhibits tumor necrosis factor-induced activation of phospholipase A2. J. Immunol. **151,** 4286–4294.

JÄÄTTELÄ, M., and WISSING, D. (1992). Emerging role of heat shock proteins in biology and medicine. Ann. Med. **24,** 249–258.

JACQUIER-SARLIN, M.R., FULLER, K., DINH-XUAN, A.T., RICHARD, M.J., and POLLA, B.S. (1994). Protective effects of hsp70 in inflammation. Experientia **50,** 1031–1038.

JUN C.D., CHOI, B.M., KIM, H.M., and CHUNG, H.T. (1995). Involvement of protein kinase C during taxol-induced activation of murine peritoneal macrophages. J. Immunol. **154,** 6541–6547.

KLEINERT, H., EUCHENHOFER, C., IHRIG-BIEDERT, I., and FORSTERMANN, U. (1996). In murine 3T3 fibroblasts, different second messenger pathways resulting in the induction of NO synthase II (iNOS) converge in the activation of transcription factor NFkappaB. J. Biol. Chem. **271,** 6039–6044.

LAEMMLI, U.K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature **227,** 680–685.

LARRICK, J.W., and WRIGHT, S.C. (1990). Cytotoxic mechanism of tumor necrosis factor-alpha. FASEB J. **4,** 3215–3223.

LIS, J., and WU, C. (1993). Protein traffic on the heat shock promoter: parking, stalling, and trucking along. Cell **74,** 1–4.

LIU, S., ADCOCK, I.M., OLD, R.W., BARNES, P.J., and EVANS, T.W. (1993). Lipopolysaccharide treatment in vivo induces widespread tissue expression of inducible nitric oxide synthase mRNA. Biochem. Biophys. Res. Commun. **196,** 1208–1213.

LOWENSTEIN, C.J., and SNYDER, S.H. (1992). Nitric oxide, a novel biologic messenger. Cell **70,** 705–707.

MAULIK, N., WEI, Z., LIU, X., ENGELMAN, R.M., ROUSOU, J.A., and DAS, D.K. (1994). Improved postischemic ventricular functional recovery by amphetamine is linked with its ability to induce heat shock. Mol. Cell. Biochem. **137,** 17–24.

MONCADA, S., PALMER, R.M., and HIGGS, E.A. (1991). Nitric oxide: physiology, pathophysiology, and pharmacology. Pharmacol. Rev. **43,** 109–142.

NARUMI S., FINKE, J.H., and HAMILTON, T.A. (1990). Interferon gamma and interleukin 2 synergize to induce selective monokine expression in murine peritoneal macrophages. J. Biol. Chem. **265,** 7036–7041.

NATHAN, C.F., and HIBBS, J.B., Jr. (1991). Role of nitric oxide synthesis in macrophage antimicrobial activity. Curr. Opin. Immunol. **3,** 65–70.

SAKURAI, H., KOHSAKA, H., LIU, M.F., HIGASHIYAMA, H., HIRATA, Y., KANNO, K., SAITO, I., and MIYASAKA, N. (1995). Nitric oxide production and inducible nitric oxide synthase expression in inflammatory arthritides. J. Clin. Invest. **96,** 2357–2363.

SCUDERI, P., STERLING, K.E., LAM, K.S., FINLEY, P.R., RYAN, K.J., RAY, C.G., PETERSEN, E., SLYMEN, D.J., and SALMON, S.E. (1986). Raised serum levels of tumour necrosis factor in parasitic infections. Lancet **2,** 1364–1365.

SNYDER, Y.M., GUTHRIE, L., EVANS, G.F., and ZUCKERMAN, S.H. (1992). Transcriptional inhibition of endotoxin-induced monokine synthesis following heat shock in murine peritoneal macrophages. J. Leukoc. Biol. **51,** 181–187.

STUEHR, D.J., and MARLETTA, M.A. (1987). Induction of nitrite/nitrate synthesis in murine macrophages by BCG infection, lymphokines, or interferon-gamma. J. Immunol. **139,** 518–525.

STUEHR, D.J., and NATHAN, C.F. (1989). Nitric oxide. A macrophage product responsible for cytostasis and respiratory inhibition in tumor target cells. J. Exp. Med. **169,** 1543–1555.

TOWBIN, H., STAEHELIN, T., and GORDON, J. (1979). Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc. Natl. Acad. Sci. USA **76,** 4350–4354.

UNANUE, E.R., and ALLEN, P.M. (1987). The basis for the immunoregulatory role of macrophages and other accessory cells. Science **236,** 551–557.

YUI, Y., HATTORI, R., KOSUGA, K., EIZAWA, H., HIKI, K., and KAWAI, C. (1991). Purification of nitric oxide synthase from rat macrophages. J. Biol. Chem. **266,** 12544–12547.

Address reprint requests to:
Dr. Han Do Kim
Department of Molecular Biology
Pusan National University
Kumjung Gu, Pusan 609-735, Korea

E-mail: hdkim@hyowon.pusan.ac.kr

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2. D EDELMAN, Y JIANG, J TYBURSKI, R WILSON, C STEFFES. 2007. Lipopolysaccharide Up-Regulates Heat Shock Protein Expression in Rat Lung Pericytes. *Journal of Surgical Research* **140**:2, 171-176. [CrossRef]
3. Mark I. Hirsh, Naoyuki Hashiguchi, Yu Chen, Linda Yip, Wolfgang G. Junger. 2006. Surface expression of HSP72 by LPS-stimulated neutrophils facilitates ##T cell-mediated killing. *European Journal of Immunology* **36**:3, 712-721. [CrossRef]
4. M SCHAFFER, U TANTRY, A BARBUL. 2004. Wound fluid inhibits wound fibroblast nitric oxide synthesis. *Journal of Surgical Research* **122**:1, 43-48. [CrossRef]
5. I Cvetkovic. 2004. 5-Aza-2#-deoxycytidine stimulates inducible nitric oxide synthase induction in C6 astrocytoma cells. *Brain Research* **998**:1, 83-90. [CrossRef]
6. X Wang. 2001. Differential Regulation of Interleukin-12 and Interleukin-10 by Heat Shock Response in Murine Peritoneal Macrophages. *Biochemical and Biophysical Research Communications* **287**:5, 1041-1044. [CrossRef]