Heat Shock Protein 70 Suppresses Astroglial-inducible Nitric-oxide Synthase Expression by Decreasing NFκB Activation*

(Received for publication, March 20, 1996, and in revised form, April 24, 1996)

Douglas L. Feinstein‡§, Elena Galea‡, Dennis A. Aquino†, Gloria C. Li, Hui Xu‡, and Donald J. Reist‡

From the 1Division of Neurobiology, Cornell University Medical College, New York, New York 10021, the 1Department of Neurology, Albert Einstein College of Medicine, Bronx, New York 10461, and the 1Radiation and Hyperthermia Biology Laboratory, Memorial Sloan Kettering Cancer Center, New York, New York 10021

In brain glial cells, expression of calcium independent nitric oxide synthase (NOS-2) is induced following stimulation with bacterial endotoxin (lipopolysaccharide (LPS)) and/or pro-inflammatory cytokines. We have investigated the effects of heat shock (HS), which can reduce inflammatory responses in several cell types, on the induction of glial NOS-2 expression. Preincubation of cells for 20–60 min at 43 °C decreased subsequent levels of NOS-2 induction, with a maximal 80% reduction after 60 min of HS. Following HS, cells were refractory to NOS inducers for up to 4 h, after which time little or no suppression was observed. HS reduced cytosolic NOS-2 enzymatic activity (3-fold), steady state mRNA levels (2–3-fold), and gene promoter activity (by 50%). HS also reduced LPS-induced nuclear accumulation of transcription factor NFκB p65 subunit, suggesting perturbation of NFκB activation.

A role for HS protein (HSP) 70 in NOS-2 suppression by HS is supported by the demonstration that 1) transfection with human HSP70 cDNA partially replicated HS effects; 2) antisense, but not sense, oligonucleotides directed against rat HSP70 partially blocked HS effects; and 3) rat fibroblasts stably expressing human HSP70 did not express NOS-2 in response to LPS plus cytokines. As with heat-shocked cells, HSP70-transfected cells also exhibited decreased NFκB p65 subunit nuclear accumulation.

In brain, inflammatory responses of astroglial cells occur during disease, infection, and ischemia. This response includes release of pro-inflammatory cytokines, such as interleukin 1-β (IL-1β)1 and tumor necrosis factor-α (TNF-α), as well as synthesis and release of nitric oxide (NO). In astrocytes, NO is biosynthesized by the calcium independent isoform of NO synthase (NOS-2) which is normally not present but whose expression is activated by a variety of inflammatory stimuli (1, 2). In vitro studies using primary rat (1, 3, 6), mouse (4), and human (5) astrocytes and glial cell lines (3, 6, 7) have demonstrated that stimulation with bacterial endotoxin lipopolysaccharide (LPS) or with a combination of cytokines which nominally includes IL-1β leads to de novo expression of NOS-2. In vivo, astroglial NOS-2 expression has been described in demyelinating diseases including experimental allergic encephalomyelitis in rodents (8) and human multiple sclerosis brain samples (9, 45), following excitatory stimulation by kainate acid (10) and following transient focal ischemia (11). In some cases, use of the selective NOS-2 inhibitor aminoguanidine can diminish the extent of damage (12), providing evidence that astroglial-derived NO contributes to pathological damage. Methods to reduce and/or prevent astroglial NOS-2 induction will therefore be of value in efforts to reduce inflammatory-related neurological damage.

Several methods have been described for regulation of NOS-2 expression in vitro (2, 13). Pretreatment of cells with anti-inflammatory agents such as dexamethasone or glucocorticoids prevents NOS-2 expression in macrophages (14) and astrocytes (6); while anti-inflammatory cytokines including IL-4, IL-10, and transforming growth factor-β1 can reduce glial and macrophage NOS-2 induction (6, 16). NOS-2 induction is potently blocked by specific inhibitors of protein-tyrosine kinases, whose activation represents an early, necessary step in the inflammatory activation of cells (17–19). A third means of glial NOS-2 regulation conceivably restricted to neural cells is via endogenous neurotransmitters. Thus activation of β-adrenergic receptors by norepinephrine reduced astroglial, but not RAW 264.7 macrophage NOS-2, expression (20). Other neurotransmitters, including ATP and glutamate, can reduce astroglial NOS-2 expression (21), whereas angiotensin II can reduce NOS-2 expression in C6 glioma (22) and smooth muscle cells (23). Finally, NOS-2 induction requires activation of transcription factor NFκB (24), and prevention of that activation prevents macrophage (24, 25), smooth muscle (26), and astroglial2 NOS-2 expression. The ability of NO itself to down-regulate NOS-2 expression (15, 27) has also been attributed to inhibition of NFκB activation (28, 29).

An additional mechanism for preventing or reducing inflammation and associated damage is the heat shock (HS) response, present in virtually all species from bacteria to human. The HS response is elicited by a variety of stimuli, including thermal, chemical, and physical stress, and it is thought that the HS

1 D. L. Feinstein, unpublished observations.
response confers resistance against subsequent and more lethal stress (30). In addition to producing a general downregulation of cellular RNA and protein synthesis, the HS response also causes rapid expression of HS proteins (HSPs) which may protect cells by facilitating renaturation of partially denatured proteins. In addition, HSPs can also restrict inflammatory responses themselves. Thus, HS expression can protect cells from inflammatory damage occurring during zymosan-induced synovitis (31), acute pulmonary inflammation (32), cardiac ischemia (33, 34), and endotoxin (35, 36). Overexpression of HSP70 also protects WEHI tumor cells against TNF-α cytotoxicity (37), rat pancreatic islet β-cells against IL-1α effects (38), inhibits LPS-induced monokine synthesis in macrophages (39), and blocks TNF-α production in retina (54). The mechanism(s) underlying these protective effects are not known, but one possibility is that HS expression can suppress NOS-2 induction, since NO may contribute to many of these events.

The effects of HS on astroglial immune responses have not been examined. However, HS expression can be induced in these cells in vitro (40, 41) as well as in vivo (42), and HS expression can be protective in several neuropathologies (30). We have therefore tested the possibility that the HS response can reduce or prevent astroglial NOS-2 expression. A recent report indicates that the HS response can reduce NOS-2 induction in rat pulmonary smooth muscle cells (67). We now show that HS reduces NOS-2 induction caused by LPS or cytokines in astrocytes and other cell types, and that this effect is mediated by expression of HSPs, notably HSP70. Finally, we present evidence suggesting that HS suppresses NOS-2 induction by interfering with NF-κB activation, suggesting a novel role for HSPs in the modulation of inflammatory responses.

EXPERIMENTAL PROCEDURES

Materials—Cell culture reagents (DMEM, antibiotics), NOS cofactors (FMN, FADH, tetrahydrobiopterin), n-butyryl coenzyme A, and LPS (Salmonella typhimurium) were from Sigma. Fetal calf serum (FCS) was from Atlanta Biological (Norcross, GA). Recombinant human TNF-α and IL-1β (both 10 units/ml) were purchased from Genzyme (Cambridge, MA). Recombinant rat IFN-γ (4 × 106 units/mg), Lipo-pectin, genitin, and Optimem were from Life Technologies, Inc. Taq polymerase was from Promega Biotech Inc. Anti-HSP70 monoclonal antibody SPA810 was from Stressgen (Victoria, Canada), and anti-NF-κB p65 polyclonal SC-109 was from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Peroxidase-conjugated goat secondary antibodies were from Vector Laboratories (Burlingame, CA). **[32P]dATP (100,000 dpm/50 μl) of assay.** PCR products were separated by agarose gel electrophoresis, bands were excised, and incorporated radioactivity was determined by scintillation counting. cDNA levels were calculated by comparison with synthesis of internal standards as described (49).

Heat Shock Procedure—Cells were incubated for the indicated times in a cell culture incubator equilibrated to 43 ± 0.5 °C, 95% humidity, and 95% O2. Following incubation, cells were returned to a 37 °C incubator and allowed to recover for desired times before inducers were added or cells were harvested for analysis.

Heat Shock Protein Analysis—Following HS, cells were allowed to recover for 30 min, after which the medium was replaced with methionine-free DMEM containing 50 μCi/ml **[35S]methionine.** After an additional 2-h incubation at 37 °C, whole cell lysates were prepared by lysis in 10% trichloroacetic acid (TCA) and 10% urea. Aliquots containing equivalent amounts of protein were electrophoresed through denaturing 10% polyacrylamide gels, the gels were dried, and incorporated **[35S]methionine** was visualized by exposure to Kodak XAR film overnight.

Stable Transfection of C6 Cells—C6 cells were plated in 60-mm dishes (at 30–40% confluency), were transfected with plasmid pCAT-NOS-2 (50) by CaPO4 precipitation, and stained for 24 h. An aliquot of cell lysate containing 20–100 μg of protein was electrophoresed through denaturing 10% polyacrylamide gels, the gels were dried, and incorporated **[35S]methionine** was visualized by exposure to Kodak XAR film overnight.

Regulation of Glial nitric-oxide Synthase 17725

**NOS-2 Activity Measurements—** NOS-2 activity was determined by measurement of NO2 in the cell culture media, from 12 to 48 h after addition of inducers. An aliquot of the media (100 μl) was mixed with 50 μl of Griess reagent (47), incubated 5 min at room temperature, and the absorbance at 546 nm was determined in a microplate reader. Standards of NaNO2 diluted in DMEM served as control.

**NOS-2 activity was measured directly in cytosolic lysates as the conversion of l-arginine to l-citrulline (1).** Assays were done in the presence of excess cofactors (FMN, FADH, tetrahydrobiopterin), 20 μM cold arginine, and 2 mM EDTA to chelate free calcium.

RNA Analysis—Total cytoplasmic RNA was prepared from cells by the Nondenat P-40 lysis procedure (48). Levels of NOS-2 mRNA were determined by a competitive reverse transcriptase-PCR assay (49). The primers used for NOS-2 detection were 1704F (5′-CTCGATGGAACAGTATAAAGGCAAAC-3′), corresponding to bases 1704–1728, and 1933R (5′-CAGACAGTCTCTGCTGATCTGAT-3′), complementary to bases 1908–1933 of rat inducible NOS cDNA sequence (49). The mRNA levels of constitutively expressed G3PDH were determined in parallel in order to normalize for cDNA synthesis efficiency. PCR conditions were 35 cycles of denaturation at 93 °C for 35 s, annealing at 63 °C for 45 s, and polymerization at 72 °C for 45 s, followed by 10 min at 72 °C. Amplifications were done in the presence of known amounts of internal deletion constructs which use the same primers as the cDNAs and (T[32P])dATP (100,000 dpm/50 μl of assay). PCR products were separated by agarose gel electrophoresis, bands were excised, and incorporated radioactivity was determined by scintillation counting. cDNA levels were calculated by comparison with synthesis of internal standards as described (49).

**C6 Cell Culture—** Primary astrocytes were prepared from cerebral cortices of postnatal day 1 Sprague-Dawley rats as described (1). At confluency, the cultures were shaken overnight to remove adhering microglial cells and used within 2–3 days for experiments. These cultures consist of greater than 95% astrocytes and between 1 and 3% microglial cells (1). C6-N cells were passaged once per week and maintained in DMEM with 10% FCS. The Rat-1 cell line and transfectants (43, 44) were grown in DMEM containing 10% FCS, and 400 μg/ml genitin was added to the transfectant cells when passaged. RAW 264.7 macrophages were grown as C6 cells.

**NOS-2 Induction Protocol—** The growth medium was removed from confluent, or near-confluent cells, the cells were washed three times in serum-free media, and then NOS-2 inducers were added in fresh serum-free media. In some experiments, 1% FCS was present with the inducers to supply exogenous LPS receptors (CD14) which confer LPS responsive-
were scraped from the plate and counted. Essentially identical results were obtained with the two methods of quantitation. In all cases, the amount of cell extract used and time of incubation were controlled to ensure that product formation remained within the linear range of the assay.

**Transient Transfections**—C6 cells were transiently transfected by CaPO4, co-precipitation with plasmid pTK-HSP70 in which expression of the human HSP70 cDNA clone pTk7.5 is under control of the herpes simplex virus thymidine kinase promoter and two copies of the SV40 enhancer. The control vector contained bacterial CAT gene in place of HSP70. Two days after transfection, the cells were analyzed for NOS-2 induction by addition of LPS plus IFN-γ.

C6 cells were transiently transfected using Lipofectin and synthetic phosphodiester oligonucleotides (ODNs). The ODNs (sense: 5′-ATG GCC AAG AAA ACA-3′; antisense: 5′-TGT TTT CTT GCC CAT-3′) flank the starting ATG codon of rat HSP70 (52) and differ in 6 of 15 bases from the constitutively expressed HSC73 (66). ODNs (25 μg/ml) plus Lipofectin (25 μg/ml) were incubated together at room temperature for 15 min in Optimem, then diluted 5-fold with Optimem before use. Subconfluent C6 cells were washed twice with Optimem, then 400 μl of diluted Lipofectin-ODN complex were added. Transfections were carried out for 8 h, then cells were washed, and fresh DMEM containing 10% FCS was added back. After an additional 16 h, the cells were incubated at 43°C for 0 or 40 min, allowed to recover at 37°C for 30 min, and then fresh medium containing LPS plus IFN-γ was added to induce NOS-2 expression, which was measured 18 to 28 h later by accumulation of NO2 in the culture media.

Preparation of Cell Extracts—Whole cell extracts were prepared for immunoblot analysis by homogenization in 8 M urea, aliquots were mixed with an equal volume of 2× SDS gel sample buffer (124 mM Tris-Cl, pH 6.8, 0.2% SDS, 10% β-mercaptoethanol, 10 mM EDTA, 50% glycerol) and boiled for 5 min. Cytosolic and nuclear extracts were obtained using a Nonidet P-40 lysis procedure (53). Cells were washed in cold PBS, collected by centrifugation (1,000 g, 4°C, 5 min), and then resuspended in 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. After a 15-min incubation on ice, Nonidet P-40 was added to a final concentration of 0.6%, the incubation continued for 15 min, and nuclei were collected by centrifugation at 10,000 × g for 15 min. The cytosolic fraction was mixed with SDS sample buffer and boiled. The nuclear pellet was washed once in lysis buffer without Nonidet P-40, then lysed in 8 M urea, and prepared with SDS sample buffer for immunoblot analysis.

Immunochromatographic Procedures—Protein samples (50–100 μg) were mixed with an equal volume of 2× SDS sample buffer, boiled for 5 min, and then separated through 8% or 10% PAGE-SDS gels. After electrophoresis, proteins were transferred to nylon membranes by semi-dry electrophoretic transfer. The membranes were blocked in 5% dry milk (1 h), rinsed, and incubated with primary antibodies (1:2000 anti-HSP70, 1:2000 rabbit anti NF-κB p65) in Tris-buffered saline (TBS) overnight at 4°C. Primary antibody was removed, membranes were washed 4 times in TBS, and 0.1 μg/ml peroxidase-labeled goat secondary antibody was added for 1 h. Following 4 washes in TBS, bands were visualized by ECL and exposure to x-ray film.

For immunostaining, cells were plated directly onto poly-lysine-coated cover slips and grown for 2–3 days in complete media. At desired times after addition of LPS plus CM, the cells were washed twice in ice-cold PBS, fixed 1 h at room temperature in 4% paraformaldehyde, washed twice in TBS, permeabilized with 0.3% Triton X-100 in TBS, and then incubated with anti NF-κB p65 antibody overnight at 4°C. The cells were washed 4 times in TBS, and signals were visualized using the peroxidase-anti-peroxidase method with an ABC staining kit (Vector Laboratories) and diaminobenzidine as chromogen. The percentage of cells which stained positively for nuclear p65 was determined by counting at least 4 different fields of 30–50 cells each, and considering only those cells with strong, clear nuclear staining as positive.

Data Analysis—All experiments were done at least three times and expressed as means ± S.E. Statistical significance was assessed by one-way analysis of variance followed by Fisher’s post hoc tests, and p values <0.05 were considered significant.

**RESULTS**

Heat Shock Reduces NOS-2 Expression—Incubation of rat astrocytes or C6 glioma cells with pro-inflammatory agents (LPS or a cytokine mixture, CM, consisting of IFN-γ, IL-1β, and TNF-α) leads to de novo expression of NOS-2 mRNA, protein, and activity, and the extent of this induction can be assessed by

**FIG. 1. Effect of heat shock duration on NOS-2 expression.** C6 cells were incubated at 43°C for the indicated times, placed at 37°C for 45 min, and then fresh medium containing NOS-2 inducers (either LPS plus IFN-γ, or cytokine mixture, CM) was added. Accumulated NO2 levels in the culture media were determined 18–24 h later using Griess reagent. Data shown are means ± S.E. of 5–7 independent determinations and are relative NO2 concentrations of heat-shocked versus control cells. *, p < 0.05 versus control cells.

NO2 accumulation in the cell culture media (1, 3, 6, 7, 49). To determine if HS could influence NOS-2 induction and/or activity, we incubated C6 cells or astrocytes for various times at 43°C, after which NOS-2 inducers were added, and NO2 levels were determined 18–24 h later (Fig. 1). HS up to 20 min did not affect NOS-2 induction. However, after a 45-min HS, subsequent C6 cell NOS-2 induction was decreased to 63 ± 9% (n = 5) when induced by CM and to 41 ± 8% (n = 7) when induced by LPS of non-heat-shocked cell induction. Essentially identical results were obtained with primary astrocyte cultures, in which a 45-min HS resulted in 75 ± 7% (CM, n = 9) and 54 ± 9% (LPS, n = 9) induction compared to controls, and maximal suppression occurred after a 60-min HS which reduced activity to 27 ± 3% of control values for either inducer. HS of glial cells for up to 60 min did not promote release of intracellular lactate dehydrogenase (data not shown). Longer incubation times at 43°C and other HS temperatures were not examined.

To determine if HS effects were long-lasting, cells were heat-shocked, then allowed to recover for various times at 37°C before the addition of NOS-2 inducers (Fig. 2). The extent of NOS-2 suppression decreased with increasing recovery time. Maximal suppression was observed if inducers were added within 15 min of HS, and no significant reduction occurred if inducers were added 4 h (or longer, not shown) after HS. Essentially identical kinetics of suppression were obtained with both astrocytes and C6 cells, and no significant differences were observed between the use of LPS or CM to induce NOS-2 expression. These results indicate that the HS-induced effects are transient. Furthermore, HS also suppressed NOS-2 expression if carried out within 4 h after addition of the inducers, whereas HS at 8 h after addition of inducers was without effect (Fig. 2, filled squares). These findings suggest that for at least 4 h after addition of LPS and/or cytokines, the pathways leading to NOS-2 expression are sensitive to HS effects.

To test the possibility that HS effects on NO2 accumulation were due to reduced intracellular substrate and/or cofactor availability, we directly measured NOS-2 activity in cytosolic lysates prepared from control or heat-shocked astrocytes that had been incubated for 20 h with LPS (added 20 min after HS). When incubated in buffer containing excess cofactors and 20 μM L-arginine, the heat-shocked lysates displayed 28 ± 4% activity compared to control cells (31 ± 5 versus 107 ± 9 pmol of L-citrulline formed per 20 min per 200 μg of protein, p < 0.05, n = 6). These results demonstrate that decreased NO2 accumulation correlated with a decrease in levels of active cytosolic NOS-2 protein, and that decreases in necessary cofactors or L-arginine do not account for HS effects.
Increased NOS-2 protein levels could be due to changes in NOS-2 mRNA levels and/or protein translation. To determine if mRNA levels were decreased, we measured NOS-2 mRNA levels in control and heat-shocked astrocytes 4 h after addition of LPS (Fig. 3), a time at which NOS-2 mRNA levels are near-maximal (49). HS decreased NOS-2 mRNA levels approximately 3-fold compared to control cells, a reduction comparable to that observed in NO2 accumulation. HS had little or no effect on levels of G3PDH mRNA, suggesting a selective suppression of newly induced gene expression.

To determine if decreased NOS-2 promoter activity could contribute to HS effects on NOS-2 mRNA, we tested HS effects on C6-1200T cells which stably express the mouse NOS-2 promoter (50) attached to the bacterial CAT reporter gene (Fig. 4). Since the majority of the NOS-2 coding region and entire 3'-untranslated region are absent from the CAT construct, any effects of HS on NOS-2 mRNA stability mediated by these regions will be eliminated. The levels of CAT activity in cytosolic lysates of heat-shocked cells were significantly decreased compared to control cells (43 ± 2% of control, n = 6–9, p < 0.005), and the extent of this reduction was comparable to, although somewhat less than, the decrease observed in NO2 accumulation measured in culture media from the same cells (23 ± 1% of control, p < 0.005). These results indicate that HS blocks steps necessary for NOS-2 promoter activation, although additional effects on mRNA stability may also contribute to the reduction in NOS-2 mRNA levels.

To determine if HS effects were cell-specific, we examined NOS-2 expression in the mouse macrophage RAW 264.7 cell line (13). RAW cells were heat-shocked for 40 min at 43 °C, allowed to recover for 10, 30, or 60 min, and then 1 μg/ml LPS was added to induce NOS-2 expression assessed 20 h later by measurement of accumulated NO2 in the media. For all recovery times, HS slightly diminished NOS-2 expression (89 ± 5%, 91 ± 2%, and 82 ± 5% of control values at 10, 30, and 60 min, respectively, n = 6 for each time point); however, only at 60 min was this decrease statistically different from control values. This demonstrates that HS can suppress NOS-2 induction in other cell types (and see below), although the extent and kinetics of suppression may depend upon the particular cell type tested.

HSP70 Mediates Heat Shock Effects on NOS-2 Expression—The HS response includes both a general down-regulation of cellular activities, including protein translation and mRNA transcription, as well as selective induction of HSP expression (30). To determine which HSPs could mediate HS effects in glial cells, we monitored the pattern of [35S]methionine incorporation in astrocytes and C6 cells following HS (Fig. 5). In astrocytes, HS stimulated the synthesis of two major protein products, the major band corresponding to HSP70, and a smaller protein corresponding to HSP32 (this band was not detected with an antibody to HSP25, data not shown). HS of C6 cells also stimulated synthesis of these two HSPs, as well as of...
proteins corresponding to HSP60, -90, and -110. Since the predominant labeled product in both cell types was HSP70, we focused attention on the possible role of this protein in mediating suppressive effects of HS on NOS-2 induction. Immunoblot analysis (Fig. 6) of whole cell extracts revealed that following HS of astrocytes, HSP70 accumulation began within 30 min, reached maximal levels between 4 and 8 h, and was still present at 24 h. Low levels of HSP70 were detectable in the non-heat-shocked cells. Similar kinetics of appearance were observed for HSP70 expression in C6 cells.

To determine if HSP70 could mediate HS effects, we heat-shocked C6 cells which had first been transfected with sense or antisense oligonucleotides (ODNs) directed against rat HSP70 mRNA. The gel shown is representative of three separate experiments.

The above results suggested that HSP70 expression could account, at least in part, for the ability of HS to reduce NOS-2 expression. To confirm this possibility, we transfected C6 cells with the human HSP70 cDNA (51) or with vector alone (Fig. 8). The cells were allowed to express for 2 days, then were treated with LPS and IFN-γ. Cells transfected with vector alone exhibited significantly reduced NOS-2 expression (53 ± 2% of control cells, n = 3, p < 0.05) possibly due to induction of endogenous rat HSPs, as previously noted (43, 44). However, transfection with the HSP70 containing plasmid led to a 2-fold further decrease in NOS-2 induction (27 ± 2% of control cells, n = 3, p < 0.05 versus vector transfected cells) suggesting that expression of human HSP70 protein in rat glial cells can also reduce NOS-2 induction.

The results obtained from transient transfection experiments may be complicated by activation of endogenous HSPs by the transfection procedures. To further confirm that HSP70 could replicate HS effects, we examined NOS-2 expression (Fig. 9) in transfected Rat-1 cells which stably express full-length human HSP70 (43, 44). Rat-1 cell lines, in contrast to astrocytes or C6 cells, required a mixture of LPS plus CM as well as the presence of 1% FCS to obtain maximal levels of NOS-2 induction (data not shown). NOS-2 expression in parental Rat-1 cells amounted to roughly 15% of that obtained with glial cells (NO2 accumulation was 11 ± 3 nmol of per 24 h per mg of protein, n = 10). As found for glial cells, prior HS treatment of Rat-1 cells reduced subsequent NOS-2 induction (to 70% of

![Fig. 5. HSP induction in astrocytes and C6 cells.](image)

![Fig. 6. Time course of HSP70 appearance in heat-shocked astrocytes.](image)

![Fig. 7. Antisense oligonucleotides to HSP70 reduce heat shock effects on NOS-2 expression.](image)

![Fig. 8. Transfection with human HSP70 reduces NOS-2 expression.](image)
control values). Neomycin selection alone caused a significant up-regulation of NOS-2 expression (MV-6 cell activity was 20 ± 6 nmoles of NO2 per 24 h per mg of protein, n = 7) possibly related to the decreased division rate of transfectants compared to Rat-1 cells (data not shown). HS of MV-6 cells reduced NOS-2 induction to 63 ± 7% of control values (p < 0.05 versus control cells, n = 3). In contrast to MV-6 cells, two independently isolated lines of Rat-1 cells stably expressing human HSP70 protein exhibited markedly reduced NOS-2 expression (in absence of HS) compared to either parental Rat-1 cells or MV-6 cells (M-21 cells, 1.7 ± 1, n = 8; M-25 cells, 3.5 ± 1, n = 4; nanomoles of NO2 per 24 h per mg of protein, p < 0.05 for both versus either Rat-1 or MV-6 cells). The expression of HSP70 in unheated M-21 and M-25 cells, and not in MV-6 cells, was confirmed by immunoblot analysis (43, 44, and data not shown). These observations demonstrate that constitutive expression of HSP70 can also prevent NOS-2 induction.

To determine if HS or HSP70 expression, achieved in the absence of thermal stress, could also reduce NOS-2 mRNA levels, we measured NOS-2 mRNA in MV-6 and M-21 cells 4 h after addition of LPS plus CM. In MV-6 cells, LPS plus CM elevated NOS-2 mRNA levels 63-fold over background levels (3,800 fg of RNA). In neither cell type were G3PDH mRNA levels altered by LPS plus CM treatment. Thus, HSP70 expression alone is sufficient to block NOS-2 mRNA accumulation.

Heat Shock Reduces NFκB Translocation—The above results suggest that heat shock or HSP70 reduces NOS-2 expression by blocking NOS-2 promoter activation. Since activation of transcription factor NFκB is necessary for NOS-2 induction (24–26), we tested if HS or HSP70 expression perturbed NFκB subunit p65 activation as assessed by nuclear accumulation (Fig. 10). In control C6 cells, incubation with LPS plus CM caused nuclear uptake of p65, beginning at 20 min (not shown), maximal at 60 min (77% of cells showed clear nuclear staining), and diminished, but still present, at 90 min (28% positive). In heat-shocked C6 cells, nuclear uptake of p65 commenced at approximately the same time as control cells (not shown), at all times examined was reduced compared to control cells (at 60 min 38% positive), and was almost absent at 90 min (< 15% positive). Similar results were obtained when comparing control to heat-shocked astrocyte cultures (not shown). In Rat-1 cells, LPS plus CM induced comparable levels of nuclear p65 staining in both MV-6 and M-21 cells when examined from 0 to 60 min after inducer addition (approximately 67% positive at 30 min in both cell types). However, at 90 min, MV-6 cells continued to have strong nuclear staining (38% positive), but M-21 cells exhibited little or no staining (< 5% positive). The presence of the p65 protein in M-21 cells rules out the possibility that lack of inducible NOS-2 expression in these cells is due to absence of p65.

To confirm that nuclear p65 levels were reduced in heat-shocked cells, as well as verify the identity of the nuclear antigen detected, we subjected cytosolic and nuclear extracts from control and heat-shocked astrocytes to immunoblot analysis (Fig. 11). In the absence of LPS, the NFκB p65 subunit was detected in the cytosolic, but not the nuclear fraction of both control and heat-shocked astrocytes (lane 2). In control cells, a 30-min incubation with LPS caused appearance of p65 in the nuclear fraction, as well as a corresponding loss from the cytoplasm (lane 1). In contrast, nuclear p65 levels were greatly reduced in heat-shocked astrocytes, although still present in the cytosol (lane 3). Together, these results support the conclusion that HS and HSP70 expression reduces NFκB p65 nuclear accumulation following stimulation with LPS and/or cytokines.

**DISCUSSION**

In this paper we demonstrate that inflammatory activation of the NOS-2 gene by either LPS or cytokines can be modulated
Regulation of Glial Nitric-oxide Synthase

Heat shock reduces nuclear NFκB p65 subunit levels.

Astrocytes were heat-shocked for 0 or 40 min at 43 °C, allowed to recover at 37 °C for 30 min, LPS was added, and nuclear and cytosolic extracts were prepared 30 min later. Equal amounts of protein (50 μg) were subjected to SDS-PAGE, and transferred proteins were analyzed by immunoblot for the presence of the p65 subunit. Similar results were obtained in two other experiments.

by the HS response. In glial cells, HS reduced NO2 accumulation, cytosolic l-citrulline formation, steady state NOS-2 mRNA levels, and NOS-2 promoter activity. That the effects of HS are mediated, at least in part, by HSP70 expression, and not due to the general down-regulation of transcriptional and translation processes that accompany HS, is supported by our findings that: 1) HSP70 is synthesized in these cells following HS; 2) antisense ODNs directed against rat HSP70 partially blocked HS effects; and 3) in glial cells and in Rat-1 fibroblasts, overexpression of HSP70, achieved in the absence of thermal stress, also reduced NOS-2 expression. HS also decreased NOS-2 expression in mouse RAW 264.7 cells and Rat-1 fibroblasts indicating that HSP70 regulation of NOS-2 expression is common to several cell types, although the magnitude of suppression varied between the three cell types examined. These findings suggest that HSPs, in addition to providing protective effects against protein denaturation, can also regulate the initiation of inflammatory events themselves.

HS reduced NOS-2 expression by blocking transcription of this gene, a conclusion supported by the observations that HS reduced steady state NOS-2 mRNA levels, promoter activity (as assessed by induction of CAT activity), and nuclear accumulation of the NFκB p65 subunit, a key step in NFκB activation and necessary for NOS-2 gene expression. Nuclear uptake of p65 was also perturbed in HSP70 expressing M21 cells; however, decreased nuclear levels were only observed at 90 min after addition of NO2 inducers, in contrast to results with heat shock which diminished nuclear p65 levels at all times examined. The mechanisms by which HS can interfere with the activation of NFκB are not yet known. However, one possibility is that HSP70, which also translocates to the nucleus, impedes NFκB nuclear translocation by competing for access to nuclear pore complexes through which NFκB is transported. Alternatively, HSP70 could impede NFκB activation by direct interaction with one (or more) of the NFκB constituents. The association of inhibitory IκB with NFκB p50 and p65 subunits occurs via interaction of IκB ankyrin domains with nuclear localization sites present in the p50 and p65 proteins. The association of NFκB with IκB ankyrin domains is tight and thus results in the inability of NFκB to translocate to the nucleus. The association of HSP70 with IκB may alter this interaction, thereby reducing NFκB nuclear translocation.

The glucocorticoid receptor (GR) resides in the cytosol as a large heteromeric complex containing two molecules of HSP90, and, upon hormone binding, dissociation of HSP90 allows the GR to move into the nucleus. Moreover, HSP70 is also a part of the GR complex in transfected Chinese hamster ovary cells, and, in recombinant human GR. Thus, the ability to regulate protein uptake into the nucleus may be a common feature of several members of the HSP family.

Whereas our results suggest that both HS and HSP70 diminish p65 nuclear uptake, others have failed to detect effects of HS or HSP70 on NFκB activation (37, 54). One factor which may contribute to this discrepancy is the time at which NFκB measurements are made. Thus, in our cells clear differences in nuclear p65 levels were observed between control and heat-shocked C6 cells at all times examined, while in Rat-1 cells differences were observed only at the 90-min time point. A second consideration is that conclusions that HSP70 does not affect transcriptional activation, based solely upon DNA shift assays (37, 54), may be complicated by the fact that transcriptionally inactive NFκB complexes lacking p65, for example p50 homodimers, can also bind to κB sites and result in decreased electrophoretic mobilities.

Another difference between our observations and others concerns the activation of the NOS-2 promoter. Our results demonstrate that expression of the bacterial CAT gene, under control of the NOS-2 promoter, is also reduced by HS (Fig. 4), suggesting that HS blocks transcriptional activity at the NOS-2 promoter. This conclusion is strengthened by our observations that HS reduced NFκB p65 nuclear uptake, which is necessary for NOS-2 promoter activation. It has recently been reported (67) that in pulmonary smooth muscle cells the HS response, achieved by incubation with sodium arsenite, abolished the increase in NOS-2 mRNA levels induced by IL-1β, with no reduction in NOS-2 promoter activity. It was concluded that arsenite-induced HS response either decreased NOS-2 mRNA stability, or that the reporter gene construct used was lacking HS-sensitive regions. Since the portion of the NOS-2 promoter used in our studies is identical to the one used by Wong et al. (67), it is likely that HS-sensitive elements are present in this region. It is conceivable that arsenite induction of HS response, unlike thermal stress-induced HSP70 expression, results in a pattern of HSP expression that does not impede NFκB activation. Alternatively, the use of stable cell lines (here) versus transient lines (Wong et al. (67)) could account for the contradictory results. Finally, it should be noted that interpretation of the experiments of Wong et al. (67), may be complicated by their observations that addition of arsenite alone (in the absence of IL-1β) was, in some experiments, also an effective inducer of NOS-2 promoter activity.

Comparison of the duration of the HS-mediated inhibition (Fig. 2) to the time course of HSP70 protein expression (Fig. 6) revealed that, in glial cells, the maximal suppressive effects of HS were obtained when NO2 inducers were added immediately or soon after HS, times at which HSP70 was not yet present. A similar discordance was observed for HS suppression of LPS-induced TNF-α mRNA increase (39), and those authors proposed that cellular events preceding HSP70 induction were responsible for inhibition of LPS effects. However, we favor an alternate explanation, namely that following addition of LPS and/or cytokines, the cascade of events leading to NOS-2 induction requires a period of time such that HSP70 is present.
at the same time an HSP70-sensitive step occurs. Consistent with this possibility are our results (Fig. 2) demonstrating that HS potently blocked NOS-2 induction even when carried out up to 4 h after the addition of LPS plus IFN-γ. Although the identity of the HSP70 sensitive step is not yet known, a likely candidate is nuclear uptake of p65, which does not commence for at least 10 to 20 min following addition of NOS-2 inducers. In this case, the initial nuclear uptake of p65 may not be impeded immediately after HS; however, HSP70 expression during the next several hours could interfere with sustained p65 uptake and thereby lead to significantly reduced final levels.

On the other hand, 4 h after HS, when HSP70 levels were at or near maximal, NOS-2 induction was no longer impeded. One factor that may help reconcile these observations is if the subcellular localization of the HSP70 protein is important to promoting suppressive effects. It is known that at early times following HS, HSP70 protein accumulates mostly in the nucleus while several hours later localization is mainly in the cytosol (56). Similar kinetics of nuclear localization are observed in heat-shocked glial cells. Thus, the window of suppression in heat-shocked cells may reflect a restricted period during which time HSP70 is in the correct subcellular location to exert suppressive effects.

Based upon our results and the above discussions, we propose the following model to explain the effects of HS on NOS-2 expression: Following HS, HSP70 expression begins within 30 min and continues to accumulate for the next several hours. During this time, stimulation with NOS-2 inducers initiates NFκB activation, resulting in gradual release of NFκB from IκB, and nuclear uptake commences within 20–30 min after stimulation. At this time, HSP70 levels are still low, so initial nuclear uptake is probably not impeded. However, within the next 30–60 min, HSP70 levels are sufficient to reduce NFκB nuclear uptake. The mechanism by which HSP70 blocks NFκB uptake is not yet known. However, the knowledge that HSP70 also enters the nucleus suggests that simple competition for nuclear pore complexes may be occurring. Alternatively, or in addition, HSP70 could bind to the NFκB complex as it dissociates from IκB, a state which may share features of a partially denatured protein. The decreased NFκB nuclear uptake results in decreased maximal levels attained as well as a shorter duration of nuclear NFκB content. As a consequence, we expect that binding of NFκB to the NOS-2 promoter is greatly diminished, thereby preventing efficient transcriptional activity and NOS-2 expression. Although the precise molecular mechanisms involved require further elaboration, this model provides a working basis for further studies of HSP70 effects on NFκB activation.

The expression of NOS-2 during brain pathologies has been suggested to contribute to the damage occurring during ischemia, demyelinating diseases, including multiple sclerosis, following excitotoxic damage, and during viral infection. In most of these pathologies, there is induction of HSPs, considered to be an internal response of neurons to protect themselves from further damage. Protective effects of HS and/or HSP70 expression in cardiac ischemia (33, 34, 52), sepsis (35, 36), and other inflammatory diseases (32, 36–38) have also been ascribed to the ability of HSPs to prevent irreversible protein denaturation. However, reports that HS, or HSP70, can block cytokine synthesis (39, 54), phospholipase A2 activation (37), and NOS-2 induction (67), together with the findings presented here, lead to the conclusion that prevention of inflammatory responses may contribute to the protective actions of HSPs. We therefore propose that expression of HSPs, and particularly HSP70, provides a novel mechanism by which cells can restrict inflammatory reactions.
Neuroimmunol. 64, 19–28
47. Green, L. C., Wagner, D. A., Glogowski, J., Skipper, P. L., Wishnok, J. S., and Tannenbaum, S. R. (1982) Anal. Biochem. 126, 131–138
48. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, 2nd Ed., pp. 192–194, Cold Spring Harbor, NY
49. Galea, E., Reis, D. J., and Feinstein, D. L. (1994) J Neurosci. Res. 37, 406–411
50. Xie, Q.-W., Whisnant, R., and Nathan, C. (1993) J. Exp. Med. 177, 1779–1784
51. Hunt, C., and Morimoto, R. J. (1985) Proc. Natl. Acad. Sci. U. S. A. 82, 6455–6459
52. Metz, R., Chi, S. H., Sayen, M. R., and Dillmann, W. H. (1994) Biochem. J. 298, 561–569
53. Schreiber, E., Matthias, P., Muller, M. M., and Schaffner, W. (1989) Nucleic Acids Res. 17, 6419
54. Simon, M., Reikvort, A., Schwarz, A., Frone, C., Luger, T., Jäättelä, M., and Schwarz, T. (1995) J. Clin. Invest. 95, 926–933
55. Baeuerle, P. A., and Henkel, T. (1994) Annu. Rev. Immunol. 12, 141–179
56. Welch, W. J., and Feramisco, J. R. (1984) J. Biol. Chem. 259, 4501–4513
57. Rice, N., and Ernst, M. (1993) EMBO J. 12, 4685–4695
58. Silver, P. A. (1991) Cell 64, 489–497
59. Dang, C. V., and Lee, W. M. F. (1989) J. Biol. Chem. 254, 18019–18023
60. Pratt, W. B. (1990) Mol. Cell. Endocrinol. 74, C69–C76
61. Kost, S. L., Smith D. F., Sullivan, W. P., Welch, W. J., and Toft, D. O. (1989) Mol. Cell Biol. 9, 3829–3838
62. Sanchez, R. R., Hirst, M., Scherrer, L. C., Tang, H. Y., Welsh, M. J., Harmon, J. M., Simons, S. S., Ringold, G. M., and Pratt, W. B. (1990) J. Biol. Chem. 265, 20123–20130
63. Diehl, E. E., and Schmidt, T. J. (1993) Biochemistry 32, 13510–13515
64. Srivivasan, G., Patel, N. T., and Thompson, E. B. (1994) Mol. Endocrinol. 8, 189–196
65. Bohan, S. P., and Yamamoto, K. (1994) The Biology of Heat Shock Proteins and Molecular Chaperones, pp. 313–334, Cold Spring Harbor Press, Cold Spring Harbor, NY
66. Sorger, P. K., and Pelham, R. B. (1987) EMBO J. 6, 993–998
67. Wong, H. R., Finder, J. D., Wasserloos, K., and Pitt, B. R. (1995) Am. J. Physiol. 6, L843