p38-dependent Enhancement of Cytokine-induced Nitric-oxide Synthase Gene Expression by Heat Shock Protein 70*

Received for publication, January 18, 2000, and in revised form, April 2, 2000
Published, JBC Papers in Press, April 14, 2000, DOI 10.1074/jbc.M000340200

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Heat shock protein (hsp) 70 protects cells against stress by means of its ability to chaperone denatured proteins and to modulate stress-activated signaling pathways. Because inflammatory processes are often accompanied by hsp expression and because stress and cytokines share several signaling pathways, we investigated the possibility that hsp70 might modulate the cellular response to cytokines. We found that stable cell clones overexpressing hsp70, or cells shortly after transfection with hsp70, produced 2 times more nitric oxide and inducible nitric-oxide synthase (iNOS) protein and mRNA in response to cytokines than control cells expressing undetectable amounts of hsp70. Since mitogen-activated protein kinases participate in the activation of iNOS by cytokines, we investigated whether hsp70 affected the activation of these signaling pathways. hsp70 overexpression led to a specific enhancement of the activation of the p38 pathway by cytokines, producing little or no effect on the activation of extracellular signal-regulated kinase or Jun N-terminal kinase. Blocking p38 activity with SB203580 totally abolished the enhancing effect of hsp70 on cytokine-induced endogenous iNOS mRNA accumulation or transcription of an iNOS promoter-driven luciferase gene, while having little effect on the cytokine response observed in control cells. We conclude that the p38 pathway acts as an enhancing factor in the activation of iNOS by cytokines and that hsp70 can modulate the cellular response to cytokines by acting on signaling elements upstream of p38.

Overexpression of hsp70‡ as a result of transcriptional activation after heat shock or genetic manipulation renders cells resistant to a variety of toxic agents including heat shock, TNFα, UV irradiation, oxygen radicals, and NO (1–4). During protein damaging treatments such as heat shock, the capacity of hsp70 to bind denatured proteins provides protection by preventing protein aggregation, accelerating refolding, and mediating degradation of damaged proteins (5–7). Hsp70 can also provide cellular protection by interfering with apoptosis induction (8, 9). One proposed mechanism involves the inhibition of the activation of the MAP kinases JNK and p38, two stress-activated protein kinases which contribute in some conditions to induction of apoptosis (10, 11).

The stress-activated protein kinases JNK and p38, together with ERK, belong to the family of MAP kinases which are involved in the cellular response to most external stimuli. Whereas ERK is preferentially activated by growth factors, JNK and p38 are most strongly activated by chemical and physical stresses and by inflammatory cytokines (12). The role of p38 in the cellular response to cytokines is particularly well documented. p38 was discovered as a major LPS-activated protein kinase in macrophages and as the target for a group of anti-inflammatory drugs which inhibit IL-1β and TNFα biosynthesis in monocytes (13, 14). p38 is activated by different pro-inflammatory agents and modulates the expression of several specific cytokines. Pharmacological or molecular inhibition of p38 results in reduced production of proinflammatory cytokines by fibroblasts and macrophages and impairs IFN-γ production in T helper 1 cells (13, 15–18). Both transcriptional and post-transcriptional regulatory mechanisms have been described. One key element of the action of p38 is its downstream target MAPKAP kinase-2, which regulates both the stability and the translation of cytokine mRNAs containing AU-rich sequences in their 3′-untranslated region (19, 20). p38 also phosphorylates and/or modulates the activity of a number of transcriptional factors involved in cytokine response such as STAT1, IFN regulatory factor-1, and NF-κB (15, 21–25).

The finding that hsp70 can modulate the activation of stress-activated signaling pathways raises the intriguing possibility that it may play a role in inflammatory and autoimmune diseases which are often associated with disregulated expression of cytokines (10, 11). A number of circumstantial evidence suggests a role of hsp in cytokine signal transduction and in the control of cytokine expression. Heat shock treatments sufficient to induce hsp accumulation reduced TNFα and IL-1β induction in monocytes after stimulation with LPS (26–28). In other studies heat shock either reduced or enhanced cytokine-stimulated NO production (29–32). On the other hand, cytokines can also induce hsp expression. In human monocytes, LPS and TNFα lead to the increased expression of hsp70 (33). A similar induction of hsp70 by cytokines has been reported in nonimmune cells such as rat pancreatic islets and cardiac myocytes (34, 35). This adds to more direct evidence indicating that hsp70, as other hsp, is involved in antigen presentation and mediates the induction of T helper 1-type cytokines in immune cells thus increasing cellular immunity (36, 37). Fi-
Hsp70 Enhances Cytokine-induced p38 Activity and iNOS Expression

nally, the detection of antibodies against hsp in some autoimmune disorders including type 1 diabetes, rheumatoid arthri-
tis, and systemic lupus erythematosus suggests that hsp are implicated in the autoimmune disease process (38, 39).

In the present study, we investigated the possible role of hsp70 as a modulator of the cellular response to cytokines
using iNOS as a model system for cytokine inducible genes. In two different rodent cell types, we showed that hsp70 increased the expression of iNOS and the production of nitric oxide. Furthermore, we provide evidence that this effect results from an hsp70-mediated enhancement of the activation of p38 by cytokines suggesting a novel role for hsp70 as a p38-dependent modulator of inflammatory responses.

**EXPERIMENTAL PROCEDURES**

Materials—[γ-32P]ATP (3000 Ci/mmol) was purchased from NEN Life Sciences Products (Boston, MA). Mouse IL-1β, H2O2, puromycin, and LPS were from Sigma (Sudbury, Ontario, Canada), and rat IFN-γ was from Genzyme (Cambridge, MA). SB203580 was from Calbiochem (San Diego, CA). Recombinant hsp27, ATF2-GST, and c-Jun-GST were pu-
rfied from *Escherichia coli* transformed with appropriate plasmids (40–42). Chemicals for electrophoresis were obtained from Bio-Rad and Fisher Scientific (Nepean, Ontario, Canada).

**Antibodies**—Anti-HA is a mouse monoclonal antibody that recog-
nizes a peptide sequence from human influenza hemagglutinin protein (Roche Molecular Biochemicals, Mannheim, Germany). The rabbit poly-
clonal antibody against iNOS was from Dianova (Hamburg, Germany).

The rabbit polyclonal antibody against hsp70 (number 799) recognizes the inducible form of hsp70 (43); anti-GST-MAPKAP kinase-2, the p45 and p54 isoforms of MAPKAP kinase-2 (44); and anti-ERK, the 14 carboxy-
terminal amino acids of ERK-2 (44). Antibodies against phosphorylated MKK3/6 and MKK4 were obtained from New England Biolabs (Beverly, MA).

**Cell Culture**—The rat insulinoma cell line RINm5F (hsp70 trans-
fected clones R70/20 and R70/3, and transfection control clone RK/1 (43)) were cultured at 37 °C in a
f10% CO2. Cells were seeded at a density of 0.5

**Immunoprecipitation and Kinase Assays**—Cells were seeded at a density of 8

**RESULTS**

**hsp70 Enhanced NO Production and iNOS Gene Expression**—To test whether hsp have any influence on the cellular response to cytokines, RIN cells were exposed to heat shock, allowed to recover for 1 or 24 h at 37 °C, and then exposed to IL-1β. The nitrite content of the supernatant was determined thereafter and used as a parameter for stimulated NO produc-
tion. Heat shock caused an immediate reduction of the ability of the cells to produce NO in response to IL-1β, but slightly enhanced this response at 24 h, at a time when the concentra-
tion of hsp70 had increased threefold (Fig. 1A).

To determine more directly whether hsp70 could be the mole-
cule responsible for the modulation of NO production, cyto-
kine-induced NO production was evaluated in RIN and WEHI
cells and compared with clones of these cell lines that consti-
tively express hsp70. Twenty-four hours after treatment
with IL-1β, RIN cell clones overexpressing hsp70 (R70/20 and
R70/3) produced a level of nitrite 2–3 times higher than con-

buffer, proteins were boiled for 5 min, electrophoresed in 10% SDS-
polyacrylamide gels, and blotted onto a nitrocellulose filter (51). After
reacting the membranes with the specific antibodies the detection step
was performed using an ECL detection kit (Amersham Pharmacia Biotech) or by iodinated secondary antibodies and quantification using the PhosphorImager analysis (Molecular Dynamics, Sunnyvale, CA).

**mRNA Isolation and RT-PCR**—Total RNA was isolated after
removal of the supernatant and lysis of the cells in Trizol (Life Technol-
gies). Specific mRNA levels were determined and quantified by RT-
PCR as described elsewhere (52, 53) using specific primers for β-actin (CLONTECH Laboratories Inc., Palo Alto, CA) and iNOS (53). RT-PCR products were labeled by hybridization to 32P-labeled probes binding at
sites between the primer sequences. iNOS mRNA levels were quantified by measuring the 32P-stimulated luminescence by PhosphorImager
analysis. Relative luminescence was calculated by normalization of the measured signals to the strength of the β-actin signals.

**Immunoprecipitation and Kinase Assays**—Cells were seeded at a density of 8 × 104/ml and incubated for at least 16 h at 37 °C. After
stimulation the cells were scraped in lysis buffer containing 20 mM
MOPS, pH 7.0, 10% glycerol, 80 mM β-glycerophosphate, 5 mM EDTA,
0.5 mM Na3VO4, 5 mM Na2P2O7, 50 mM sodium fluoride, 1% Triton X-100, 1 mM benzamidine, and 1 mM dithiorthreitol, and 1 mM phenylmethylsulfonyl fluoride. After vortexing, the extracts were cen-
trifuged at 17,000 × *g* for 10 min at 4 °C and the supernatants were
incubated on ice. The immunoprecipitations were performed using
the supernatants were diluted four times in buffer A (20 mM Tris-HCl, pH
7.5, 150 mM NaCl, 0.1 mM EDTA, 1 mM EGTA, 1 mM MgCl2, 1 mM
Na3VO4, 1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride). Undi-
luted anti-ERK2, anti-MAPKAP kinase-2, or anti-HA-tag antibodies
were added in limiting concentrations. After 1 h, protein A-Sepharose
(Amersham Pharmacia Biotech, 50% v/v in buffer A) was added for
another 30 min. The samples were centrifuged for 15 s and washed
three times with 300 μl of buffer A. Immunoprecipitates were used
directly for kinase assays. MAPKAP kinase-2, ERK2, and p38 activities
were determined in immune complexes using appropriate substrates.

**MAPKAP kinase-2 activity** was determined using recombinant hsp27
as substrate (44). The assays were done in 20 μl of buffer K (100 μM
ATP, 30 μM MgCl2, 5 mM MnCl2; pH 7.0, 10% glycerol, 15 mM MgCl2, 0.05% Triton X-100, 1 mM dithiothreitol, 1 μM leupeptin, 0.1 mM phenylmethylsulfonyl fluoride). The kinase assay was assayed for 30 min at 30 °C and stopped
by boiling in SDS sample buffer. Immunoprecipitated ERK2 and p38
were assayed analogously using myelin basic protein and GST-ATP-2, re-
spectively, as substrates. Kinase assay buffer K containing 10 mM
MgCl2 was used for ERK2. The assay buffer for p38 contained 50 μM
Hepes, pH 7.4, 50 mM β-glycerophosphate, 50 mM MgCl2, 0.2 mM
Na2VO4, 4 mM dithiothreitol, A2P2GST, and γ-32P[ATP. In the case of
JNK, the cell extract was adsorbed on GST-Jun beads and the kinase
test using the same GST-N-terminal Jun as substrate (42). Briefly,
the GST-Jun fusion protein was incubated for 30 min at 4 °C with
the extracts. The beads were then pelleted, washed, and incubated for
30 min at 20 °C with [γ-32P]ATP in buffer K containing 10 mM
MgCl2. The phosphorylated GST-Jun was boiled in SDS sample buffer
to stop the reaction. The activity of the various kinases was quantified
by measuring the incorporation of radioactivity into the specific sub-
strate by PhosphorImager analysis after electrophoresis.
control-transfected (RK/1) or wild type RIN cells (Fig. 1B). Similarly, treatment with LPS and IFN-γ resulted in a 3-fold increased nitrite production in the hsp70-overexpressing WEHI cell line Wn113-5 as compared with the empty vector transfected Wn10x cells (Fig. 1C). In both cell lines, the time course analysis revealed not only an increase but also a more rapid induction of NO production. Since NO activity depends on the specific signaling pathways used by these stimuli.

**hsp70 Enhanced p38 MAP Kinase Activation**—MAP kinases are in many cell lines important modulators of iNOS gene induction. We analyzed the activity of the MAP kinases p38, JNK, and ERK after stimulation with LPS and IFN-γ in control versus hsp70 overexpressing WEHI cell clones. Hsp70 caused a major change both in the kinetics and strength of p38 activation measured either by the *in vivo* activation of its downstream target MAPKAP kinase-2 (Fig. 3) or directly by measuring the activity of immunoprecipitated p38 (data not shown). A maximal difference in activity was observed at 10 min after exposure, at which time MAPKAP kinase-2 activity was induced about 12-fold in hsp70-overexpressing WEHI cells as compared with 2-fold in control cells. A similar hsp70-dependent enhancement of MAPKAP kinase-2 activation was observed in WEHI or RIN cells exposed to IL-1β (see below and data not shown). LPS/IFN-γ induced no significant ERK activity in either control or hsp70-expressing WEHI cells. JNK was strongly activated by this treatment, however, in several experiments no or little difference was obtained between control and hsp70-expressing cells.

The effect of hsp70 expression on MAP kinase activation was reproduced in a transient transfection assay. Parental WEHI cells were transfected with HA-tagged p38 or HA-tagged JNK constructs together with increasing concentrations of the hsp70 expression vector. Twenty-four hours later the cells were treated with LPS/IFN-γ for 15 min and the activity of the transfected kinase was determined after immunoprecipitation with a limiting concentration of a HA antibody. A progressive enhancement of p38 activation was obtained with increasing concentrations of hsp70. Furthermore, transfection of a hsp70 antisense construct in hsp70 overexpressing WEHI clones reduced the activation of HA-p38 kinase. At similar concentrations, hsp70 had no effect on the activation of HA-tagged JNK (Fig. 4).

The hsp70-specific enhancement of p38 kinase activation in response to LPS/IFN-γ contrasted with previous results showing an inhibition of stress-induced p38 and JNK activation by hsp70 (10, 11). We therefore determined the effect of hsp70 on the activation of p38 by IL-1β as compared with activation by two toxic stressing treatments, hydrogen peroxide and heat shock. Treatment of hsp70-expressing WEHI cells with IL-1β led to the same enhancing effect on kinase activity compared with control cells as did the treatment with LPS/IFN-γ. In contrast, elevated expression of hsp70 led to a reduced hydrogen peroxide and heat shock activation of p38 (Fig. 5). This result suggests that the effect of hsp70 on p38 activation depends on the nature of the activating pathway used by the stimuli.

Three MAP kinase kinases, MKK4, MKK3, and MKK6, can regulate the activation of p38 (41, 54–56). We therefore looked at the effect of hsp70 on the activation (phosphorylation) of these kinases by LPS/IFN-γ and hydrogen peroxide (Fig. 6). A major difference was found in the induction of phosphorylation of MKK4 and MKK3/6 by the two stimuli. Hydrogen peroxide treatment led to the phosphorylation of both MKK3/6 and MKK4, although the former was induced more strongly. In contrast, LPS/IFN-γ treatment induced a strong phosphorylation of MKK4 but no significant increase in the phosphorylation of MKK3/6. As found at the level of p38, hsp70 caused an enhancement in the cytokine-induced phosphorylation, but a reduction in the stress-induced phosphorylation of the MAP kinase kinases, hence suggesting that hsp70 acts further upstream in the specific signaling pathways used by these stimuli.

**The Enhancing Effect of hsp70 on iNOS Expression Is p38 Dependent**—The transcription of the iNOS gene is regulated by several transcriptional factors some of which have been shown...
to be regulated by p38. To verify whether the effect of hsp70 on iNOS expression was due to enhanced transcription, RIN cells were transfected with pNOS-1002LUC, a reporter gene containing the first 1002-base pair upstream sequence of the rat iNOS gene fused to luciferase. As reported previously, this construct contained all necessary information for proper activation by cytokines (47). Upon expression of the reporter gene in RIN cells, a 10-fold increase in luciferase activity was obtained after stimulation with IL-1β. The same cells also transfected with hsp70 showed a 30-fold increase in luciferase activity, hence, a 3-fold increase in activation relative to control cells (Fig. 7A). No effect of hsp70 expression was observed on the constitutive expression of β-galactosidase driven by the cytomegalovirus promoter (data not shown).

This result clearly showed that the effect of hsp70 was at the level of the iNOS gene promoter. To verify whether p38 was responsible for the enhancing effect of hsp70 on the transcriptional activation of iNOS, the cells were exposed to IL-1β in the presence of a specific p38 inhibitor (see Fig. 7B). As expected, the inhibitory effect of the inhibitor was also observed in RIN/70/20 cells, indicating that p38 was indeed a critical component in mediating the effect of hsp70 on iNOS expression.

**Fig. 2.** Effect of hsp70 on cytokine-induced iNOS mRNA and protein expression. A, control Wn10x cells (open circles) and hsp70-expressing Wn113-5 cells (filled squares) were treated with IFN-γ (100 units/ml) and LPS (100 ng/ml). At various times thereafter, total RNA was isolated and the iNOS and β-actin mRNA amplified by RT-PCR. The amounts of iNOS mRNA were quantified by PhosphorImaging and values of phosphostimulated luminescence (PSL) were calibrated to the amount of β-actin mRNA. Shown are means of three experiments ± S.D. B, Wn10x and Wn113-5 cells were treated as in A; RIN and R70/20 cells were treated with IL-1β (10 units/ml). Total protein lysates were prepared 24 h later and analyzed by Western blot to determine iNOS protein expression.

**Fig. 3.** Effect of hsp70 expression on cytokine-induced MAP kinase activity. Control Wn10x cells (open circles) and hsp70-expressing Wn113-5 cells (filled squares) were treated with IFN-γ (100 units/ml) and LPS (100 ng/ml). At various times thereafter, cell extracts were prepared and processed to determine the activity of MAPKAP kinase-2 (MAPKAP-K2, A), JNK (B), and ERK (C) using the appropriate proteins as substrate. The radiolabeled substrates were then separated by electrophoresis and the kinase activities visualized by autoradiography and quantified by PhosphorImager analysis. The relative induction of kinase activity was calculated by setting the value for untreated controls as 1. Graphs on the left are from average values of two experiments. Representative autoradiograms are shown on the right.
The inhibition of p38 by SB203580 only slightly reduced the luciferase activity in control cells but completely abolished the enhancing effect of hsp70 on iNOS-promoter driven luciferase activity (Fig. 7A). A similar inhibitory effect of SB203580 was obtained on the expression of endogenous iNOS mRNA in control and hsp70-

FIG. 4. Effect of hsp70 concentration on cytokine-induced p38 kinase activity. A-C, Wn10x cells were co-transfected with HA-tagged p38 together with varying concentrations of hsp70 (pZhsp70tag) or control (pZEMneo) plasmids. Twenty-four hours later, cells were treated (+) with IFN-γ (100 units/ml) and LPS (100 ng/ml) for 15 min or left untreated (−) and HA-tagged p38 was immunoprecipitated to determine p38 activity using ATF2-GST as substrate. Shown are the calculated relative induction of kinase activity (A), the autoradiogram of labeled ATF2 (B), and for each condition the level of hsp70 in cell extracts determined by Western blot analysis (C). D and E, cells were processed as above except that they were co-transfected with HA-tagged JNK. The relative induction of immunoprecipitated HA-JNK was calculated (D) after measuring the level of phosphorylation c-Jun-GST used as substrate (E). F and G, HA-tagged p38 activity in Hsp70-overexpressing Wn113-5 cells which were co-transfected with HA-tagged p38 together with antisense-hsp70 (ashsp70) plasmids at the indicated concentrations and stimulated as above. Both relative induction (F) and ATF2 phosphorylation (G) are shown. Similar results were obtained in three separate experiments.

FIG. 5. Effect of hsp70 expression on the induction of MAPKAP kinase-2 activity by different treatments. Control Wn10x cells (open circles and hsp70 − ) and hsp70-expressing Wn113-5 cells (filled squares and hsp70 + ) were treated with 100 units/ml IL-1β (A and B), 1 mM H2O2 (C and D), or heat shock at 43 °C (E and F). MAPKAP kinase-2 was immunoprecipitated at the indicated times after initiating the treatments and its activity measured using hsp27 as substrate. The relative induction of MAPKAP kinase-2 (A, C, and E) was calculated from the autoradiograms shown in B, D, and F, setting the value of untreated controls as 1. Shown is one of three representative experiments.

presence of the p38 inhibitor SB203580. The inhibition of p38 by SB203580 only slightly reduced the luciferase activity in control cells but completely abolished the enhancing effect of hsp70 on iNOS-promoter driven luciferase activity (Fig. 7A).
expressing WEHI cells. SB203580 had little if any effect on the iNOS mRNA content of control WEHI cells after stimulation with LPS/IFN-γ (Fig. 7B), but completely abolished the hsp70 enhancing effect on iNOS expression. These results indicated that p38 can up-regulate cytokine-induced iNOS gene transcription and that an increased activation is responsible for the observed enhancing effect of hsp70.

DISCUSSION

The work presented in this study stemmed from the observation that heat shock, while causing an initial inhibition of cytokine-induced NO production, resulted at 24 h post-treatment in an enhanced response to cytokines. An inhibition of the cytokine response early after heat shock has been observed previously in different cell types such as rat lung, liver, smooth muscle cells, or glial cells (29–31), and likely results at least in part from initial heat-induced damage. Protein denaturation during heat shock is the trigger for hsp gene transcription. Accumulation of chaperone hsp which bind denatured proteins and assist refolding, facilitates cell recovery. Eventually, accumulation of hsp is also responsible for turning off hsp transcription (57). In the present study, we presented strong evidence that elevated expression of hsp70 after heat shock can modify the cellular response to cytokines. RIN and WEHI cells overexpressing hsp70 produced 2 to 3 times more NO than control cells after treatment with IL-1β or a combination of LPS and IFN-γ, respectively. The enhanced NO production did not result from a mere stabilization of iNOS activity through hsp70 chaperone function. Hsp70-mediated enhancement of NO production was accompanied by an increased transcriptional activity of the iNOS gene and an increased accumulation of iNOS mRNA and protein. Furthermore, the effect appears specific to hsp70, since overexpression of another hsp, hsp27, produced no enhancement of NO production (data not shown).

Besides the well documented hsp70 chaperone activity (5–7) which likely contributes to its protective functions during a number of toxic conditions and mediators such as heat shock, TNFα (1), reactive radicals (4, 58), or UV light (3), hsp70 also mediates in normal physiological conditions numerous cellular activities including protein synthesis, folding, transport, and degradation (6, 59–61). Here we found that hsp70 expression enhanced LPS/IFN-γ or IL-1β-induced p38 MAP kinase activation in WEHI cells. A similar effect was also observed in RIN cells exposed to IL-1β (data not shown). The effect of hsp70 on p38 activation was concentration dependent. It increased proportionally with the concentration of hsp70 in transiently transfected cells. Furthermore, in cells constitutively expressing hsp70, the enhanced p38 activation could be abrogated by expression of a hsp70 antisense construct. The effect was also specific to p38. Hsp70 overexpression had no effect on cytokine-induced JNK or ERK activation. Finally the effect was specific to cytokines. In the same cells, hsp70 antagonized hydrogen peroxide or heat shock-induced p38 activation.

The mechanisms responsible for the opposite effect of hsp70 on stress activation versus receptor-mediated activation of the p38 signaling pathway is unclear. In the case of stress activation, it is conceivable that the hsp70 inhibitory effects result from an inhibition of the initial damaging event that is responsible for triggering p38 activation. For example, hsp70 was shown to block apoptosis and p38 can be activated downstream of some apoptotic events (9, 62). The findings that hsp70 also reduced activation of the p38-activating kinases MKK3/6 and MKK4 support the view that hsp70 does not act directly on p38 but instead at a level more proximal to the initial triggering event. Thus the action of hsp70 on p38 activation is different from its action on JNK. The inhibition of JNK activation by hsp70 was reported to result from a protection mediated by hsp70 at the level of a JNK phosphatase that is sensitive to protein damaging agents (63). In that study, the mechanism for the inhibition of p38 activation was not identified but was shown to be different. In contrast, in the case of receptor-mediated activation of p38, a chaperone activity of hsp70 at the level of signaling elements that contribute positively to p38 activation may prevail. There are a few reports indicating an association of hsp70 with the proximal elements of signaling pathways other than p38. For example, hsp70 together with hsp90 associate with the inactive glucocorticoid receptor, keeping it in a competent state for activation (64). Hsp70 is also associated with the scaffold complex formed of the kinase suppressor of Ras (KSR), the ERK MAP kinase kinases MEK1 and MEK2 and other proteins (65). Hsp70 was found to bind and increase the stability of the MAP kinase kinase kinase MOS (66). Similarly, elevated expression of the chaperone hsp70...
may stabilize or promote the formation of signaling complexes linking cytokine receptors to the p38 activation cascade, resulting in an enhanced activation. Our results showing that hsp70 enhances the activation of p38-activating kinases support such an action of hsp70 early in the signaling pathway.

The study also indicated that the increased activation of p38 was responsible for the hsp70-mediated enhancement of cytokine-induced iNOS expression. The additional iNOS mRNA which accumulated in a hsp70-dependent manner was abolished in the presence of the p38 inhibitor SB203580. Interestingly, p38 activity was not found to play a major role in the basal expression of iNOS in control cells but was responsible for the enhancing effect observed in hsp70-transfected cells. Similar results were obtained in the RIN cell model stimulated with IL-1β. In these cells overexpression of hsp70 enhanced cytokine-induced transcription of a luciferase reporter gene containing the cytokine-regulated elements of the rat iNOS gene. The enhancement was totally abolished in the presence of the p38 inhibitor, suggesting that the hsp70-mediated effect occurred at the level of transcription and was in major part regulated by p38.

The role of the MAP kinases and in particular p38 in modulating iNOS expression has been investigated in several studies. Varying results have been obtained which likely resulted from the different nature of the cell lines and agonists used, but also from the complexity of the regulatory mechanisms of iNOS regulation and the multitude of targets of p38 which can influence iNOS expression. In macrophages activated by LPS/IFN-γ, as we found here in control WEHI cells, inhibition p38 activity had little effect on iNOS expression (67, 68). In most cases, however, as we found here in the hsp70 overexpressing cells, inhibition of p38 led to a partial inhibition of the response (23, 69–72). The murine iNOS promoter contains several regulatory elements, of which at least three bind transcriptional factors known to be regulated by the p38 pathway and to be essential for full expression of iNOS. The iNOS promoter contains two NF-κB binding motifs. Upon activation, NF-κB translocates to the nucleus and binds to DNA. p38 has little effect on the binding activity but contributes to NF-κB mediated transactivation (15, 24, 25). The most distal region of the iNOS promoter contains 2 regions directly and indirectly regulated by STAT1: an IFN-stimulated response element which binds IFN regulatory factor-1, a factor which is newly synthesized in cells after stimulation with LPS/IFN-γ (22) and requires STAT1 for its own transcriptional activation, and a IFN-γactivated site which binds STAT1 dimers directly (73). Targeted disruption of the STAT1 gene abolishes completely the response to IFN-γ (74). Recently it has been shown that p38 plays a key role in the phosphorylation of STAT1 at serine 727 and the activation of its transcriptional activity (21, 75). Thus, p38 activity might enhance iNOS transcription directly through STAT1 activation at the IFN-γ-activated site but also indirectly at the IFN-stimulated response element since blocking p38 activity impairs STAT1 dependent accumulation of IFN regulatory factor-1 after IFN-γ stimulation (22). In many of these studies, inhibition of p38 activity led to partial inhibition whereas constitutive activation of p38 led to a major enhancement of the response. Thus any factor which like hsp70 can enhance p38 activation would be expected to increase importantly the expression of iNOS. Intriguingly, an insulin-dependent enhancement of iNOS gene expression coupled with an enhancement of p38 activation was also observed in glomerular mesangial cells stimulated with IL-1β (76).

NO is an important mediator in inflammation and autoimmune diseases (49, 77), acting at high concentrations as a cytotoxic agent and at lower concentrations as an immunoregulatory molecule suppressing T-helper 1-type cytokines such as IFN-γ and increasing T-helper 2-associated molecules such as IL-4 (78). iNOS is only one of several immunoregulatory genes that are modulated by p38 activity. Pharmacological or molecular inhibition of p38 have revealed important roles of p38 both in the inflammatory response of macrophages and fibroblasts and in the response of T-helper-1 effector cells (13, 15–18, 20). Hsp70 expression is increased in most cell types after stress, during inflammatory or autoimmune processes, and in some cells at specific stages of differentiation (57). The finding that hsp70 can modulate the activation of p38 in response to cytokines and thereby influence the activation of cytokine-regulated genes may reveal important consequences of stress in inflammatory and immune processes.

Acknowledgments—We thank R. M. Tanguay, J. Grose, J. Moscat, M. Jäättela, J. Woodgett, and D. L. Eizirik for providing several of the antibodies and reagents used in this study.
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J. Biol. Chem. 2000, 275:18172-18179.
doi: 10.1074/jbc.M000340200 originally published online April 14, 2000

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