Induction of cyclooxygenase-2 by heat shock protein 60 in macrophages and endothelial cells

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The 60-kDa heat shock protein (HSP60) has been identified as a danger signal to the innate immune system (6). It appears to be a key endogenous inflammatory mediator generated in response to tissue injury and/or stress and is presumably released by damaged cells. HSP60 stimulates macrophages to produce cytotoxic and proinflammatory mediators, including nitric oxide, tumor necrosis factor (TNF)-α, interleukin (IL)-6, and IL-12 (6, 29). HSP60 functions by activating the toll-like 4 receptor, a transmembrane protein that is also important in intracellular signaling initiated by lipopolysaccharide (LPS) from gram-negative bacteria (1, 4, 26, 39, 41, 47, 52–54). Similarities in the intracellular domain structure of the toll-like 4 receptor and the IL-1 receptor form the basis of current models to explain the mechanism of action of HSP60 and LPS (39). Engagement of the toll-like 4 receptor causes the adapter protein MyD88 to associate with the receptor (27, 42, 63). This results in activation of an IL-1 receptor-associated kinase (IRAK), followed by phosphorylation and activation of TNF receptor (TNFR)-associated factor (TRAF)-6 (20, 34, 48, 62). This pathway is known to be critical for the activation of nuclear factor (NF)-κB, as well as the mitogen-activated protein (MAP) kinases, signaling molecules that are important in the transcriptional regulation of many cytotoxic and proinflammatory mediators in macrophages, including the inducible form of nitric oxide synthase (NOS-2) (7, 15, 52, 54).

It is well recognized that macrophages release lipid mediators, including prostaglandins (PGs) and leukotrienes that promote inflammation (32, 37, 45). The synthesis of PGs is dependent on the activity of cyclooxygenase (COX), an oxidoreductase that converts arachidonic acid into the common PG precursor, PGH2 (61). Two isoforms of the enzyme have been identified, a constitutive form referred to as COX-1 that is expressed in most cell types, and COX-2, an inducible form that is thought to be important in inflammation (22, 55). Macrophages express COX-2 in response to a variety of cytokines as well as LPS (22, 37, 68). In the present studies, we determined if COX-2 was also induced by HSP60. We found that HSP60 was an effective inducer of COX-2 in macrophages, as well as...
endothelial cells. Moreover, activation of MAP kinases, c-Jun/cAMP-response element (CRE) binding protein (CREB), and NF-κB signaling pathways are important in this activity. These data provide additional support for the idea that HSP60 is important in nonspecific host defense and can act as an endogenous mediator of inflammation.

MATERIALS AND METHODS

Chemicals. Recombinant mouse and human interferon (IFN)-γ were kindly provided by Dr. S. Pestka, University of Medicine and Dentistry of New Jersey (UMDNJ)-Robert Wood Johnson Medical School (Piscataway, NJ). LPS derived from Escherichia coli (serotype O55: B5) was purchased from Sigma Chemical (St. Louis, MO). Recombinant human HSP60 (lot no. 008404) and antibodies against p65 Rel A were from StressGen Biotechnologies (Vancouver, BC). 1,2-N'-iminoethyloornithine (NIO) was obtained from Alexis Biochemicals (San Diego, CA) and 4-(5-(chloromethyl)-5-(trifluoromethyl)-1H-pyrazol-1-yl)benzenesulfonamide (SC-236) from Calbiochem (La Jolla, CA). Antibodies against NOS-2, p50, COX-2, CREB, c-Jun, and CCAAT enhancer binding protein (C/EBP)-β and all secondary antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies against extracellular signal-regulated kinase 1/2 (ERK1/2), phospho-ERK1/2, c-Jun-NH2-terminal kinase (JNK)/stress-activated protein kinase (SAPK), phospho-JNK/SAPK, p38 MAP kinase, and phospho-p38 MAP kinase were from Cell Stress-activated protein kinase (SAPK), phospho-JNK/SAPK, p38 MAP kinase, and phospho-p38 MAP kinase were from Cell Signaling Technology (Beverly, MA). 4-(4-Fluorophenyl)-2-(4-methylsulfonylphenyl)-5-(4-pyridil)1H-imidazole (SB-203580) and 2-(2-amino-3-methoxyphenyl)1H-benzo[4-1one (PD-98059) were from Calbiochem (La Jolla, CA). Unless otherwise indicated, all other chemicals were from Sigma Chemical.

Cells, treatments, and measurements of nitric oxide production. Primary cultures of rat endothelial cells and alveolar macrophages were prepared as described previously (16, 43). RAW 264.7 mouse macrophages were from Dr. D. Wolff, UMDNJ–Robert Wood Johnson Medical School (Piscataway, NJ). Cells were cultured in DMEM supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin. Nitric oxide production by the cells was measured by the accumulation of nitrite in the culture medium using the Greiss reagent with sodium nitrite as standard (21). For these studies, cells (5×10^6) were plated into 24-well plastic culture dishes. Each well contained 0.3 ml of culture medium. After 24 h, the medium was changed to serum- and phenol red-free growth medium without and with appropriate concentrations of HSP60 (1–10 μg/ml). Several experiments were performed to exclude the possibility that LPS contamination was responsible for the biological effects of HSP60 (6, 30). Using a Limulus amoebocyte lysate assay (E-toxase kit, Sigma), we found that treatment medium with HSP60 contained <0.08–0.09 ng/ml of LPS; concentrations well below those required to induce COX-2 and NOS-2 in the cells (data not shown). Moreover, LPS but not HSP60-induced expression of COX-2, as well as NOS-2 and nitric oxide production, was inhibited by the addition of polymyxin B (up to 30 μg/ml) to the cultures. The activity of HSP60, but not LPS, was also found to be heat labile. Table 1 summarizes the effects of polymyxin B and heat treatment on nitric oxide production induced by HSP60 in RAW264.7 macrophages. In these studies, polymyxin B (0.3 and 3 μg/ml) reduced LPS- but not HSP60-induced nitrite accumulation in the culture medium of the macrophages. Conversely, heat treatment (85°C, 15 min) inhibited the activity of HSP60, but not LPS. These data indicate that the biological effects of HSP60 were not due to LPS contamination.

Western blotting. Methods for the preparation of cell lysates and Western blotting have been described previously (21). Briefly, cells were solubilized in buffer containing 1% Triton X-100, 10% glycerol, 50 mM Tris·HCl, pH 7.4, 50 mM NaCl, 0.1 mM sodium orthovanadate, 0.1 mM phenylmethylsulfonyl fluoride, 5 μg/ml aprotinin, and 5 μg/ml leupeptin. Equal amounts of protein (10–30 μg/ml) were electrophoresed on 7.5 or 10% polyacrylamide gels, transferred to nitrocellulose membranes, and then probed with a specific primary antibody followed by a horseradish peroxidase-conjugated secondary antibody. A chemiluminescence-based detection kit was used to visualize protein expression (Renaissance Plus, Perkin-Elmer Life Sciences).

RNA isolation and RT-PCR of COX-2. RNA was isolated from the macrophages using Trizol reagent (GIBCO BRL, Rockville, MD) according to the manufacturer’s instructions. First-strand cDNA synthesis was performed with SuperScript II for RT-PCR (GIBCO BRL) using 0.4 μg RNA in each reaction. PCR was run using 20 ng cDNA, COX-2 mouse upstream primer (5′-CATTCTTGGCCAGACCTCATTCC3′) and downstream primer (5′-GACCCAGGACCCAGACAGGACGACGAC3′; Ambion Gene-Specific Relative RT-PCR kit), and Taq DNA polymerase (GIBCO BRL). PCR amplification was performed using a modification of the manufacturer’s protocol to allow for COX-2 quantification using 18S as an internal standard. Varying cycles were used to optimize quantitation. The PCR products were amplified using a 1-min hot start at 94°C and 22 cycles of 30 s at 94°C, 30 s at 56°C, and 60 s at 72°C and a final extension at 72°C for 10 min. Products were separated by electrophoresis using 8% urea polyacrylamide gels followed by autoradiography.

Transfection of macrophages. Reporter plasmids driving the expression of firefly luciferase fused to either wild-type or mutated promoter constructs of the murine COX-2 gene were kindly provided by Dr. H. Herschman, Univ. of California (Los Angeles, CA). Mutated promoter constructs for the CRE (mCRE), E-box (mE-box), NF-κB (mNF-κB), and two NF-IL-6 (mNF-IL-6–1, mNF-IL-6–2) sites were used (68). The reporter construct in which the mutated NF-IL-6 site was located further upstream from the start of the luciferase gene was designated mNF-IL-6–1, while the mutated site further downstream was designated mNF-IL-6–2 (68). To assess
transfection efficiency, cells were cotransfected with a Renilla-luciferase cDNA. Plasmids were introduced into cells by electroporation using a Bio-Rad Gene Pulser II system (200 V, 1,000 μF) and then seeded into 24-well culture plates (10^6 cells/well). After incubation at 37°C for 24 h, the cells were treated with HSP60 (10 μg/ml). Luciferase activity was then assessed 6 h later with a Turner model 20/20 luminometer by measuring the conversion of a luciferase-sensitive substrate to a luminescent species (Dual Luciferase Assay, Promega). Results are reported as the ratio of firefly luciferase activity to Renilla luciferase activity in the extract.

Electrophoretic mobility shift assay. Nuclear extracts were prepared as described by Schreiber et al. (59). Oligonucleotide consensus sequences for either NF-IL-6 (5'-TGCAGAT-TGGCCGCAATCTGCA-3'), CRE (5'-TCCCATGGAGATCATG-GTTT-3'), NF-κB (5'-AGTTGAGGGACTTCCAGGGC-3'), or IFN-γ activating sequence (GAS) (5'-GATCGATTCCCGAAT-3') (10, 13, 40, 70) were labeled with 32P and incubated at room temperature for 30 min with 15 μg of nuclear protein. Binding reactions were performed in a total volume of 25 μl in a buffer containing 5 mM HEPES, pH 8.0, 5% glycerol, 1 mM MgCl2, 40 mM NaCl, 0.05 mM dithiothreitol, 0.4% BSA, and 0.1 μg/ml salmon sperm DNA. In competition experiments, nonradioactive oligonucleotide was added to the binding reaction mixture in a 100:1 ratio relative to labeled oligonucleotide. The protein/DNA complexes were then fractionated on 4.5 or 7% polyacrylamide gels. Gels were transferred to Whatman filter paper, covered with plastic wrap, dried for 1 h, and then exposed to Kodak MS film (−70°C, 18 h). For p65 Rel A, c-Jun, CREB, or c/EBP-β supershifts, nuclear extracts were incubated with a 1:50 dilution of anti-p65 Rel A, anti-c-Jun, or anti-CREB, or a 1:5 dilution of anti-c/EBP-β antibodies, respectively, for 30 min on ice before addition of the labeled oligonucleotide.

RESULTS

Effects of HSP60 on COX-2 expression. The present studies report for the first time that HSP60 is an effective inducer of COX-2 in macrophages and endothelial cells. In both cell types, HSP60 readily induced COX-2 protein expression in a concentration- and time-dependent manner (Fig. 1, A and B, top; Fig. 2, A and B, and not shown). Whereas in the macrophages, expression of COX-2 was observed within 4 h, in endothelial cells, it was delayed for 16 h (Fig. 1, A and B, top). In macrophages, induction of COX-2 protein was found to be due to increased steady-state expression of COX-2 mRNA (Fig. 2D).

Previous studies have demonstrated that many inducers of COX-2 also upregulate the expression and activity of NOS-2 (32, 37, 45, 61). We found that HSP60 readily induced nitric oxide production in both macrophages and endothelial cells and was as effective as LPS (Fig. 1, A and B, bottom). The combination of LPS and HSP60 was, in general, more effective in inducing nitric oxide production than LPS alone. The time course of HSP60-induced NOS-2 expression was similar to induction of COX-2 in both cell types (Fig. 1, A and B, top). Induction of COX-2 and NOS-2 by HSP60 was also observed in normal primary cultures of alveolar macrophages, indicating that the effects of HSP60 were not limited to a macrophage tumor cell line (data not shown).

In macrophages, maximal induction of NOS-2 occurs when cytokines such as IFN-γ are combined with LPS (50). Similarly, we found that treatment of the macrophages with the combination of IFN-γ and HSP60 caused a significantly greater induction of nitric oxide production (Fig. 3, top) and NOS-2 compared with either agent alone (Fig. 3). In contrast, IFN-γ had no effect on HSP60-induced COX-2 expression in the cells (Fig. 3, bottom). These data suggest that there are distinct mechanisms regulating expression of COX-2 and NOS-2 in macrophages.

Previous studies have suggested that the activity of NOS-2 may be important in regulating the expression of COX-2 (23, 38, 56). We found that the well-characterized NOS-2 inhibitor NIO did not alter HSP60-

![Fig. 1. Heat shock protein 60 (HSP60) induces cyclooxygenase (COX)-2 and nitric oxide synthase (NOS)-2 in macrophages and endothelial cells. Cells were treated without or with HSP60 (10 μg/ml) and/or lipopolysaccharide (LPS; 10 ng/ml) as described in MATERIALS AND METHODS. Cell lysates were probed for COX-2 and NOS-2 expression by Western blotting (A and B, top) and culture supernatants were analyzed for nitrite content (A and B, bottom). Data represent means ± SE of triplicate samples. ● Control; ○, LPS; ▼, HSP60; v, LPS + HSP60.](c14181f01a)
induced COX-2 expression (Fig. 2C). Similarly, SC-236, a specific inhibitor of COX-2, had no effect on either the expression of NOS-2 or production of nitric oxide by the cells (Fig. 2C and not shown). These data indicate that in mouse macrophages, neither COX-2 nor NOS-2 activity is required for HSP60-induced expression of these proteins and further support the idea that their expression is regulated independently.

**Regulation of HSP60-induced COX-2 expression.** To examine regulatory elements required for HSP60-induced transcriptional activation of COX-2, we transfected macrophages with wild-type or mutated COX-2 promoters fused to a luciferase reporter gene. The wild-type mouse promoter contains two NF-IL-6 regulatory regions (mNF-IL-6–1, mNF-IL-6–2) and one site for the E-box, CRE, and NF-/H9260/B elements (68). HSP60 was found to readily induce luciferase activity in cells transfected with the wild-type COX-2 reporter. Activity was significantly decreased in HSP60-treated cells transfected with mutated constructs for NF-/H9260/B, CRE, or NF-IL-6 (Fig. 4). Interestingly, the two NF-IL-6 response elements were not equally active. Compared with the mutated site near the 5'-end of the promoter (mNF-IL-6–1), mutation of the site near the 3'-end (mNF-IL-6–2) resulted in a sharper decline in HSP60-induced luciferase activity. The luciferase activity in extracts from cells transfected with mNF-IL-6–2 alone. These data demonstrate a role for the NF-κB, CRE, and NF-IL-6 regulatory elements in transcriptional activation of COX-2 and also emphasize the importance of the location of these elements within the promoter in controlling COX-2 expression.

On the basis of these results, we next examined the activity of transcription factors that bind to the regulatory elements that appear to be involved in induction of COX-2 by HSP60. Using mobility shift assays, we found that HSP60, like LPS, readily activated NF-/H9260/B in the cells (Fig. 5B). The combination of HSP60 and LPS did not further increase NF-κB activity (data not shown). Western blot analysis confirmed nuclear translocation of both the p50 and p65 subunits of NF-κB after treatment of cells with HSP60 (Fig. 5A). CREB and c-Jun bind CRE regulatory regions in responsive gene promoters (13, 60). HSP60, like LPS, readily activated CRE in macrophages. Supershift assays confirmed the presence of both CREB and c-Jun in the CRE binding complex (Fig. 6A). These
data are in accord with the transfection studies described above and further demonstrate the importance of NF-\(\kappa\)B, C/EBP, and c-Jun/CREB in regulating HSP60-induced expression of COX-2.

As indicated above, HSP60-induced expression of NOS-2, but not COX-2, was enhanced by IFN-\(\gamma\). To determine if HSP60 modulated IFN-\(\gamma\)-induced signaling for NOS-2, we examined DNA binding of the transcription factor signal transducer and activator of transcription (STAT)-1 to the GAS. This is a key element regulating NOS-2 gene expression (44). STAT-1 binding to GAS was detected in nuclear extracts from macrophages stimulated with IFN-\(\gamma\), but not with HSP60 (Fig. 6B). The STAT-1::GAS complex was supershifted with antibodies to STAT-1 (data not shown). HSP60 had no additional effect on IFN-\(\gamma\)-induced STAT-1 binding activity (Fig. 6B). These data indicate that HSP60 does not function by activating STAT-1 and that other transcription factors are responsible for enhancing NOS-2 expression in cells treated with the combination of HSP60 and IFN-\(\gamma\).

**Role of MAP kinases in HSP60-induced COX-2 expression.** Binding of LPS to the toll-like 4 receptor leads to activation of the MAP kinase pathways, including the p44/p42 MAP kinase (ERK1/2), JNK, and p38 kinase, each of which has been implicated in the regulation of COX-2 (2, 11, 17, 31, 46, 68). These enzymes can directly phosphorylate and activate transcription factors and/or other intracellular substrates such as protein kinases that result in transcription factor activation (31, 49). For example, the JNK kinase phosphorylates c-Jun on its NH\(_2\)-terminal activating domain, resulting in the formation of c-Jun-c-Fos heterodimers and c-Jun homodimers, which can upregulate genes via AP-1 and CRE regulatory elements (31, 49). The p38 kinase has also been reported to activate a number of transcription factors, including CREB and c-Jun (31, 49), while the activity of ERK1/2 has been reported to be important in activation of NF-\(\kappa\)B in the COX-2 promoter (5). We found that the macrophages constitutively expressed ERK1/2 kinase, JNK kinase, and p38 kinase (Fig. 7, B and C, and Fig. 8, B and E). HSP60 caused a rapid time-dependent phosphorylation of each of these proteins (Fig. 7A and Fig. 8, A and E). ERK1/2 was phosphorylated, presumably, through the action of MAP kinase kinase (MEK) 1 and MEK2 (31). PD-98059, a selective inhibitor of these kinases (2), completely blocked HSP60-induced phosphorylation of ERK1/2 (Fig. 7C). PD-98059 also caused a...
Fig. 5. Activation of NF-κB and CCAAT/enhancer binding protein (C/EBP) by HSP60 in macrophages. Macrophages were treated without or with HSP60 (10 μg/ml) for increasing periods of time (15–45 min). LPS (1 μg/ml) was used as a positive control. Nuclear extracts were prepared and analyzed by Western blotting or in mobility shift assays as described in MATERIALS AND METHODS. In mobility shift assays, the first lane of each gel shows the probe (PROBE) run in the absence of nuclear extract and the second lane (CTL), nuclear extract from buffer-treated control cells. In these gels, lanes marked CC are nuclear extracts analyzed in the presence of a ×100 excess of appropriate unlabeled probe. A: Western blot of nuclear extracts showing expression of the p50 and p65 subunits of NF-κB in control (CTL) and HSP60-treated cells. B: mobility shift assay for NF-κB. The p50 and p65 lanes show supershifts using antibodies to these proteins. B, inset: another sample analyzed with the p65 antibody that more clearly demonstrates a supershift. The p50 supershifted band was not readily visible due to its spreading over the higher molecular weight ranges in the gel. C: Western blot of nuclear extracts showing expression of C/EBP-β in CTL and HSP60-treated cells. D: mobility shift assay for C/EBP. SS, supershift with an antibody to C/EBP-β.

Fig. 6. Effects of HSP60 on CRE binding protein (CREB), c-Jun, and signal transducer and activator of transcription (STAT)-1α. Macrophages were treated for 15 min without or with HSP60 (10 μg/ml), IFN-γ (100 U/ml), or LPS (1 μg/ml) as a control. Nuclear extracts were then assayed for transcription factor activity. The first lane in each panel represents labeled probe in the absence of extract, whereas the lanes labeled CC were nuclear extracts analyzed in the presence of a ×100 excess of the appropriate unlabeled probe. Control (CTL) lanes were from cells treated with buffer alone. A: c-Jun/CREB. For supershift assays, extracts were treated with specific antibodies to CREB or c-Jun. B: STAT-1α.
marked decrease in HSP60-induced COX-2 mRNA and protein expression as well as NOS-2 protein expression (Fig. 2D and Fig. 7C). SB-203580, a selective inhibitor of the p38 kinase (11), blocked p38 phosphorylation in the macrophages (Fig. 8A). However, in contrast to the ERK1/2 inhibitor, the p38 kinase inhibitor was found to block HSP60-induced expression of COX-2, but not NOS-2 (Fig. 8, C and D).

**DISCUSSION**

The present studies demonstrate that HSP60 is an effective inducer of both COX-2 and NOS-2 in macrophages and endothelial cells. These enzymes are responsible for the production of cytotoxic and inflammatory mediators, and their ability to be induced by HSP60 provides support for the idea that this stress protein is an important endogenous activator of innate immunity (1, 6, 47). Increased expression of COX-2 in response to HSP60 was associated with an increase in steady-state expression of COX-2 mRNA. In many cells, increases in COX-2 mRNA are due to upregulation of COX-2 gene transcription (22). However, it should be noted that the COX-2 message can also be regulated posttranscriptionally. For example, the 3'-untranslated region of the murine COX-2 message contains multiple regulatory elements controlling mRNA stability and translational efficiency (9). Our studies also showed that macrophages and endothelial cells were distinct with respect to the timing of their responses to HSP60. Thus macrophages responded much more quickly to induction of COX-2 and NOS-2 than did endothelial cells, which may reflect the different origins and functions of these cells.

Within each cell type, the kinetics of expression of COX-2 and NOS-2 were similar, suggesting that there are common aspects in the signaling pathways mediating induction of these proteins. HSP60 binds the toll-like 4 receptor, a transmembrane protein with an IL-1 receptor-like intracellular domain (47). Activation of this receptor causes it to associate with MyD88 and activate several receptor-associated kinases, leading to the induction of the MAP kinase signaling cascades that participate in regulating COX-2 and NOS-2 (25, 27, 42, 63). Binding of HSP60 to the toll-like 4 receptor also activates transcription factors known to be important in regulating expression of COX-2 and NOS-2, including NF-κB (1, 4, 47). In our studies, transfection of the macrophages with reporter constructs for cis-acting elements of the 5'-flanking sequence of COX-2 revealed that NF-κB was required for optimal HSP60-induced expression of COX-2. Similar results were observed with CRE and two NF-IL-6 sites. In contrast, mutation of an E-box site in the COX-2 promoter had no effect on HSP60-induced reporter activity. Consistent with these findings, mobility shift assays showed that HSP60 readily induced NF-κB and CRE binding activity in the macrophages, while C/EBP, which binds to NF-IL-6, was constitutively active in the cells. The activated NF-κB complex was comprised of both the p50 and p65 subunits of this transcription factor while both c-Jun and CREB bound to the CRE, and C/EBP-β bound to NF-IL-6. These data indicate that NF-κB, C/EBP-β, c-Jun, and CREB play a role in HSP60-induced expression of COX-2. C/EBP-β has also been reported to be involved in the transcriptional activation of COX-2 in activated murine osteoblasts (67) and mouse skin carcinoma cells (28). In macrophages, modulation of the activity of C/EBP-β also alters COX-2 reporter activity, further demonstrating the importance of this transcription factor in regulating COX-2 expression (68). Constitutive activity of C/EBP-β has been observed in many cell types (13, 33, 51, 64). Transcriptional activity apparently requires phosphorylation (33, 51), and it is possible that HSP60 induces signaling kinases that activate this transcription factor in macrophages.

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At least six enhancer elements have been identified that regulate maximal induction of NOS-2, including two IFN-stimulated response elements, a GAS element, two NF-κB elements, and an octamer DNA sequence motif (OCT) site, the latter binding the basal transcriptional element octomer (44). Thus, at the present time, it appears that NF-κB is critical in mediating induction of both COX-2 and NOS-2 by HSP60, although it is likely that additional common regulatory elements for these two genes will be identified as the fine structure of the promoter elements are further characterized (44, 61). Previous studies using the same reporter constructs indicated that the CRE and NF-IL-6 sites, but not NF-κB, are required for optimal activity of the COX-2 promoter after LPS treatment of RAW 264.7 macrophages (68). In these cells, inhibition of LPS-induced activation of NF-κB by expression of an inhibitor NF-κB-IκB also had no effect on LPS-dependent COX-2 reporter activity (64). On the basis of these data, it appears that HSP60 and LPS induce COX-2 by distinct mechanisms. That NF-κB is not required for endotoxin-induced activation of COX-2 in RAW 264.7 cells was surprising since both HSP60 and LPS utilize the toll-like 4 receptor (30, 47, 54). Moreover, as indicated above, we found that HSP60 and LPS were equally effective in inducing NF-κB in RAW 264.7 macrophages, and LPS was able to induce COX-2. Vabulas et al. (65) also reported that HSP60 activates IκB kinase, causing degradation of IκB-α and inducing an NF-κB luciferase reporter in RAW 264.7 macrophages. Induction of NF-κB by HSP60 has also been described in human endothelial cells (29). Moreover, in many cell types, NF-κB has been reported to mediate COX-2 induction, for example, by endotoxin in J774 macrophages (12, 66) and differentiated U937 cells (24) and by TNF-α in MC3T3-E1 cells (71) and in gastric AGS cells (35). Thus it appears that there may also be distinct mechanisms for transcriptional regulation of COX-2 in different cell lines.

Our data demonstrate that HSP60 readily activates the stress-activated protein kinases JNK and p38 and the MAP kinases ERK1/2 in RAW264.7 macrophages. Specific inhibitors of the p38 kinase or ERK1/2 kinase inhibited HSP60-induced COX-2 expression, indicating that these enzymes are required for the process. Kol et al. (30) also reported activation of the p38 kinase by HSP60 in human peripheral blood mononuclear cells. SB-202190, a selective p38 MAP kinase antagonist, inhibited IL-6 production in response to HSP60 (30). The ERK1/2 kinase inhibitor used in the present studies also inhibited expression of HSP60-induced NOS-2, suggesting that HSP60 signaling via these kinases also regulates expression of this enzyme. In contrast, an inhibitor of the p38 kinase had no effect on HSP60-induced NOS-2, indicating that there are distinct mechanisms regulating HSP60-induced expression of COX-2 and NOS-2. Taken together, these data suggest that p38 kinase mediates multiple functions of HSP60. Recently, Vabulas et al. (65) demonstrated that HSP60-induced activation of JNK proceeds via the toll-like 4 receptor and involves MyD88 and TRAF-6. In addition, the signaling pathway involved required
receptor-mediated endocytosis of HSP60 in a process inhibited by serum components (65). Endocytosis of the receptor-ligand complex may be a mechanism for downregulating toll-like 4 receptors and suppressing the innate immune response in the presence of excess HSP60.

Several laboratories have suggested that NOS-2 activity is essential for cytokine and LPS induction of COX-2 or vice versa, although many of these studies are conflicting (23, 38, 50, 56). For example, NOS inhibitors have been found to suppress PG production in LPS-treated rats (56). In human fetal fibroblasts, nitric oxide treatment enhances IL-1-induced PG production (57), while peritoneal macrophages from NOS-2 knockout mice produce significantly less PGE₂ when challenged with LPS and IFN-γ (38). In contrast, nitric oxide has been reported to inhibit COX-2 expression and PGE₂ release in LPS-stimulated mouse macrophages (8), whereas inhibition of NOS has been reported to enhance COX-2 protein expression and PG production in rat peritoneal macrophages (18). These data suggest that COX-2 activity can be regulated by nitric oxide, but the specific biological response may depend on the animal and/or tissue model used. In RAW264.7 macrophages, we found that HSP60-induced expression of COX-2 was unaltered by an inhibitor of NOS-2. Similarly, an inhibitor of COX-2 did not affect HSP60-induced expression of NOS-2. These data indicate that, after HSP60 treatment, COX-2-generated prostanoids are not required for expression of NOS-2. Similarly, nitric oxide induced by HSP60 does not appear to regulate expression of COX-2.

It should be noted that COX-2 and NOS-2 are not always coordinately expressed. For example, we found that IFN-γ can selectively induce NOS-2, but not COX-2, in the macrophages. IFN-γ, but not HSP60, also induces NOS-2 in PAM 212 keratinocytes (unpublished observations). These findings are presumably due to the activation of transcription factors by IFN-γ, but not HSP60, that are important in regulating expression of this enzyme in these two cell types. This idea is supported by our findings that IFN-γ was effective in inducing phosphorylation, nuclear translocation, and DNA binding of STAT-1 in macrophages while HSP60 was inactive. IFN-γ was also found to enhance HSP60-induced expression of NOS-2 and nitric oxide production without altering COX-2. Presumably, transcription factors induced by IFN-γ can synergize with those induced by HSP60 for maximal expression of NOS-2 (29, 30, 47). This synergism may be due to simultaneous activation of the NF-κB and Janus kinase (JAK)-STAT pathways, proteins which may physically interact to augment NOS-2 transcription (44, 58).

In summary, we have demonstrated that HSP60 is an effective inducer of COX-2 and NOS-2 in macrophages and endothelial cells, providing additional evidence that this protein has the capacity to function as a proinflammatory mediator. On the basis of our data, we propose a model for the action of HSP60 (Fig. 9).

According to this model, HSP60 released during injury and inflammation binds to receptors on cells in surrounding tissues (19, 30, 36, 47, 54, 65). These receptors include those associated with the actions of endotoxin as well as α3β1 integrin (3). This in turn causes activation of the transcription factor NF-κB. HSP60 also activates p38, ERK1/2, and JNK MAP kinases. This results in activation of additional transcription factors important in the regulation of COX-2 and NOS-2, including CREB, C/EBP, and c-Jun. It is likely that additional transcription factors regulating expression of COX-2 and NOS-2 are also activated by HSP60. Expression of COX-2 requires the ERK1/2 kinase and p38 kinase. Two lines of evidence suggest that HSP60-
induced signaling leading to expression of COX-2 and NOS-2 occurs via distinct mechanisms. First, as indicated above, IFN-γ synergizes with HSP60 to induce NOS-2, but not COX-2; second, p38 kinase activity appears to differentially regulate COX-2 and NOS-2 gene expression. Synthesis of COX-2 and NOS-2 leads to production of prostanooids and nitric oxide, respectively, which contribute to and amplify the inflammatory process. Further studies that more precisely define the signaling pathways induced by HSP60 in macrophages are needed to better elucidate mechanisms regulating expression of COX-2 and NOS-2 in tissues.

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