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Pharmacological modulation of heat shock factor 1 by antiinflammatory drugs results in protection against stress-induced cellular damage

(indomethacin/cytoprotection/heat shock response)

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ABSTRACT **The activation of heat shock genes by diverse forms of environmental and physiological stress has been implicated in a number of human diseases, including ischemic damage, reperfusion injury, infection, neurodegeneration, and inflammation. The enhanced levels of heat shock proteins and molecular chaperones have broad cytoprotective effects against acute lethal exposures to stress. Here, we show that the potent antiinflammatory drug indomethacin activates the DNA-binding activity of human heat shock transcription factor 1 (HSF1). Perhaps relevant to its pharmacological use, indomethacin pretreatment lowers the temperature threshold of HSF1 activation, such that a complete heat shock response can be attained at temperatures that are by themselves insufficient. The synergistic effect of indomethacin and elevated temperature is biologically relevant and results in the protection of cells against exposure to cytotoxic conditions.**

Nonsteroidal antiinflammatory drugs (NSAIDs) are widely used in the treatment of inflammation and associated diseases (1). The primary action of these drugs is thought to occur by inhibiting the cyclic pathway from arachidonate to eicosanoids, a potent family of signaling molecules, through inactivation of prostaglandin endoperoxidase synthase (2, 3). In addition to its role as an antiinflammatory drug, salicylic acid affects the transcription of injury-response genes in plants (4), inhibits the activation of NF-κB (5), and induces heat shock transcription factor 1 (HSF1) in human cells (6). Additional support for a potential link between inflammation and the heat shock response is suggested by the ability of arachidonic acid to modulate the DNA-binding and transcriptional activities of HSF1 (7).

The elevated expression of genes encoding heat shock (stress) proteins and molecular chaperones has been detected in numerous disease states, including ischemia, reperfusion damage, oxidant injury, cardiac hypertrophy, fever, inflammation, bacterial and parasitic infection, metabolic diseases, neoplasia, and in cell and tissue damage (8–16). The activities of heat shock proteins and the heat shock response in general are likely to exert themselves at multiple levels, by serving to detect the onset of physiological stress, to prevent subsequent damage resulting from the synthesis and accumulation of nonnative proteins, and as a key component of cellular repair processes following injury (reviewed in refs. 8 and 17–25). Heat shock proteins and molecular chaperones, whether induced by prior exposure to nontoxic stress conditions or by overexpression of genes encoding heat shock proteins, have been shown to protect cells against a broad range of toxic conditions, including oxidative stress, tumor necrosis factor α (TNF-α), extreme temperatures of heat shock, ethanol, heavy metals, and cellular damage following ischemia or sepsis-induced injury (26–29).

Our rationale to look further into a link between heat shock and inflammation was based on observations that diverse conditions of cell and tissue damage resulted in the elevated expression of heat shock proteins, together with data to indicate that exposure of cells to certain prostaglandins, sodium salicylate, and arachidonic acid led to the activation of HSF1 (6, 7). HSF1 is the primary stress-inducible transcription factor that senses and responds to a wide range of conditions of physiological and environmental stress (30, 31). The process of HSF1 activation involves multiple steps, including translocation into the nucleus, oligomerization from monomer to trimer, acquisition of DNA-binding activity, inducible serine phosphorylation, and attenuation of the transcriptional response (32–39). In this study, we examine the effects of the antiinflammatory drug indomethacin and demonstrate that indomethacin pretreatment lowers the temperature threshold of HSF1 activation, such that a complete heat shock response can be attained at temperatures that are by themselves insufficient. The synergistic effect of indomethacin and elevated temperature is biologically relevant and results in the protection of cells against exposure to cytotoxic conditions.

MATERIALS AND METHODS

Cell Culture Conditions and Thermotolerance Assay. HeLa S3 cells were grown to a density of 2–4 × 10^5 cells per ml in spinner culture flasks containing Joklik's modified minimal essential medium supplemented with 5% (vol/vol) calf serum and the antibiotic gentamicin. Other human tissue culture cells including Jurkat T lymphocytes, U937 monocytes, and K562 erythroleukemia cells were grown in RPMI 1640 medium supplemented with 10% (vol/vol) heat-inactivated fetal calf serum. All cells were maintained at 37°C in an atmosphere of 5% CO_2/95% air. Cell viability was assessed by staining with trypan blue (Sigma). Indomethacin (Sigma) was dissolved in 100% ethanol to a concentration of 0.05 M and added to cultures of HeLa S3 cells at a density of 4 × 10^5 cells per ml in Joklik's medium containing 20 mM Hepes, pH 7.8. For heat shock, the cells were exposed to elevated temperatures in constant-temperature water baths.

Thermotolerance experiments were performed on HeLa cells either left untreated or exposed to various pretreatments (heat shock, indomethacin, or indomethacin and various elevated temperatures) prior to the exposure to the lethal chal-

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Abbreviations: NSAID, nonsteroidal antiinflammatory drug; HSF*n*, heat shock transcription factor *n*.

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lenge temperature of 44.5°C for 30 min. Cell survival was measured by clonal analysis (29); cells were plated in triplicate onto 6-cm dishes, and colonies containing >60 cells were counted after 7 days.

Gel Mobility-Shift Assay, Northern and Western Blot Analysis. HSF1 DNA-binding activity was analyzed by using the gel mobility-shift assay as described (39). The intensity of the shifted radiolabeled human heat shock element oligonucleotide indicating the levels of HSF1–heat shock element complexes was quantified on a PhosphorImager 400A (Molecular Dynamics). Northern blot analysis was performed by using the human *HSP70* gene radiolabeled with ^{32}P by using the Klenow fragment of DNA polymerase I as described (40). Western blot analysis for HSF1 was performed on whole cell extracts (10 μg) from control, heat shocked, and indomethacin-treated cells by using rabbit polyclonal anti-HSF1 antiserum (38). The immune complexes were analyzed with the enhanced chemiluminescence detection system (Amersham).

RESULTS AND DISCUSSION

To examine whether indomethacin, a member of the cyclic acetic acid class (including sulindac and tolmetin) of NSAIDs, leads to activation of HSF1, HeLa cells were treated with a range of drug concentrations, and the level of HSF1 was determined by gel mobility-shift analysis. As shown in Fig. 1*A*, indomethacin treatment results in activation of HSF DNA binding (lanes 5 and 6). To establish which member of the HSF family (HSF1 or HSF2) was activated by indomethacin, whole-cell extracts were preincubated with antibodies specific for either HSF1 or HSF2 and analyzed by gel mobility-shift assay. The indomethacin-induced HSF formed a higher-order complex only upon incubation with anti-HSF1 antibodies (Fig. 1*A*, lanes 7 and 8) and not with either anti-HSF2 antibodies (lanes 9 and 10) or preimmune serum (data not shown; ref. 11), thus indicating that indomethacin induces HSF1 DNA-binding activity, the same factor induced by heat shock. Pretreatment with cycloheximide did not interfere with the ability of indomethacin to induce HSF1 DNA binding (data not shown). The profile of HSF1 activation exhibits a dose–response profile with relatively rapid kinetics followed by gradual attenuation (Fig. 1*B*). The concentration of indomethacin required for HSF1 activation varies widely among human cell lines, with Jurkat T-lymphocyte cells exhibiting the highest level of drug sensitivity, K562 cells an intermediate response (similar to HeLa cells), and U937 monocytes being the least sensitive to the drug.

Indomethacin treatment activates HSF1, resulting in the conversion of the latent monomer non-DNA-binding form to the trimeric DNA-binding state. To establish whether indomethacin treatment, by itself, led to a corresponding increase in *HSP70* message levels and HSP70 synthesis, RNA was isolated for Northern blot analysis and cells were labeled with [^{35}S]methionine and analyzed by SDS/PAGE. Whereas

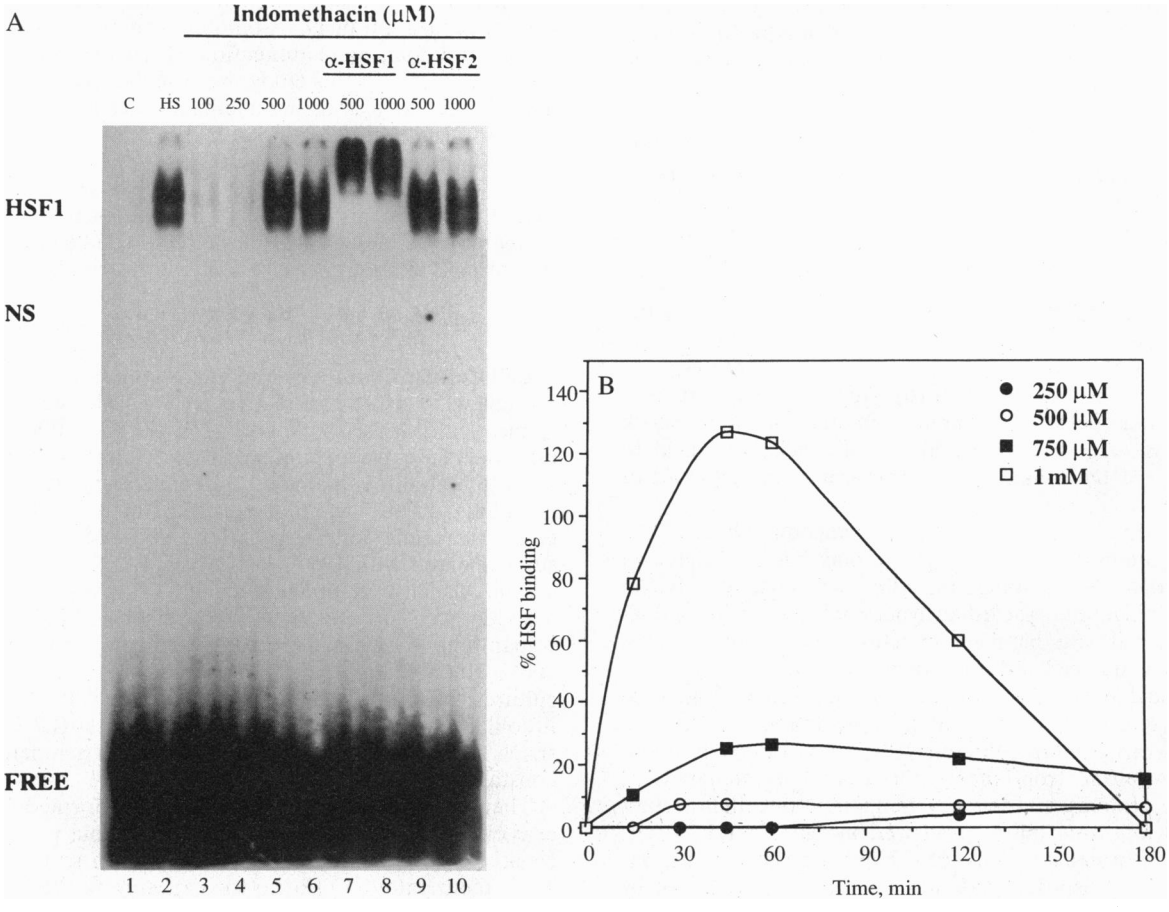

FIG. 1. Indomethacin induces HSF1 DNA-binding activity. (*A*) Gel mobility-shift analysis of whole-cell extracts from control, heat-shocked, and indomethacin-treated HeLa S3 cells after antibody detection with anti-HSF1 (α-HSF1) or anti-HSF2 (α-HSF2) polyclonal antibodies. HeLa S3 cells were treated for 60 min with 42°C heat shock as a positive control (HS), 37°C as a negative control (C), or different concentrations (100 μM to 1 mM) of indomethacin at 37°C. The whole-cell extracts were preincubated with anti-HSF1 or anti-HSF2 polyclonal antibodies, and gel mobility-shift analysis was performed. NS, nonspecific protein–DNA interaction; FREE, unbound heat shock element oligonucleotide. (*B*) Scanning densitometric analysis of the levels of HSF1 DNA binding for different concentrations of indomethacin at 37°C. HeLa S3 cells were treated with 250 μM, 500 μM, 750 μM, or 1 mM indomethacin, and whole-cell extracts were collected at intervals for gel mobility-shift analysis. The level of HSF1 was quantified by scanning densitometry and normalized to the maximal level of HSF1 DNA-binding activity after a 60-min, 42°C heat shock.

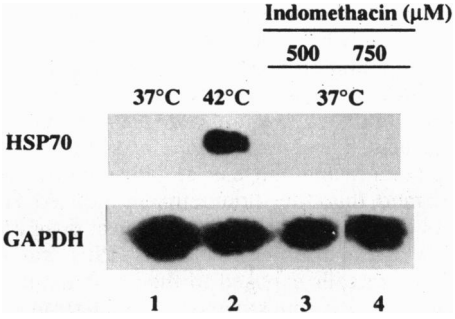

FIG. 2. Indomethacin treatment does not result in elevated levels of *HSP70* mRNA. Northern blot of RNA isolated from control, heat-shocked, and indomethacin-treated cells. Cytoplasmic RNA was isolated from HeLa S3 cells treated for 60 min with 42°C heat shock (as positive control), 37°C (as negative control), and 500 or 750 μM indomethacin at 37°C. The RNA blot was hybridized with the *HSP70* probe, stripped, and reprobed with a cDNA probe for the non-heat-shock-inducible gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) to confirm equivalent levels of mRNA in each lane.

HSP70 messenger RNA and HSP70 synthesis was readily detected in cells exposed to heat shock (Fig. 2, lane 2), neither was detected in the indomethacin-treated cells (lanes 3 and 4 and data not shown). Likewise, indomethacin treatment alone does not induce heat shock gene transcription as measured by nuclear run-on analysis (data not shown). Taken together, these data reveal that indomethacin treatment activates HSF1 DNA-binding activity without inducing a complete heat shock response.

The sites of tissue inflammation are often associated with local increases in temperature; therefore, we examined whether indomethacin could act in synergy with suboptimal heat shock temperatures to affect the temperature at which HSF1 was activated. HeLa cells exposed separately to either 40°C (Fig. 3*A*, lane 6) or 41°C (lane 10) or incubated with low concentrations of indomethacin (100 μM, lane 3, or 250 μM, lane 4) did not induce HSF1 DNA binding. However, simultaneous exposure to indomethacin and 40°C resulted in activation of HSF1 at 250 μM indomethacin (Fig. 3*A*, lane 8), whereas at 41°C, HSF1 was activated in the presence of 100 μM indomethacin (lane 11). These results reveal that indomethacin lowers the temperature threshold for induction of HSF1 activation and, furthermore, that the synergistic effect involves both reduced temperatures and lower drug levels. An additional effect of coexposure to indomethacin and 41°C was the prolonged activation of HSF1 (Fig. 3*B*). Cotreatment of HeLa cells with indomethacin and 41°C resulted in activation of HSF1 DNA-binding activity extending through 240 min (Fig. 3*B*, lanes 9–16), whereas HSF1 attenuated during exposure to the drug or heat shock alone (Fig. 1*B*; ref. 11). Therefore, the combination of indomethacin and slightly elevated temperatures has dramatic effects on both the kinetics and maintenance of HSF1 DNA-binding activity.

One interpretation of these observations is that indomethacin by itself incompletely induces the heat shock response. At the higher drug concentrations, HSF1 is induced to acquire

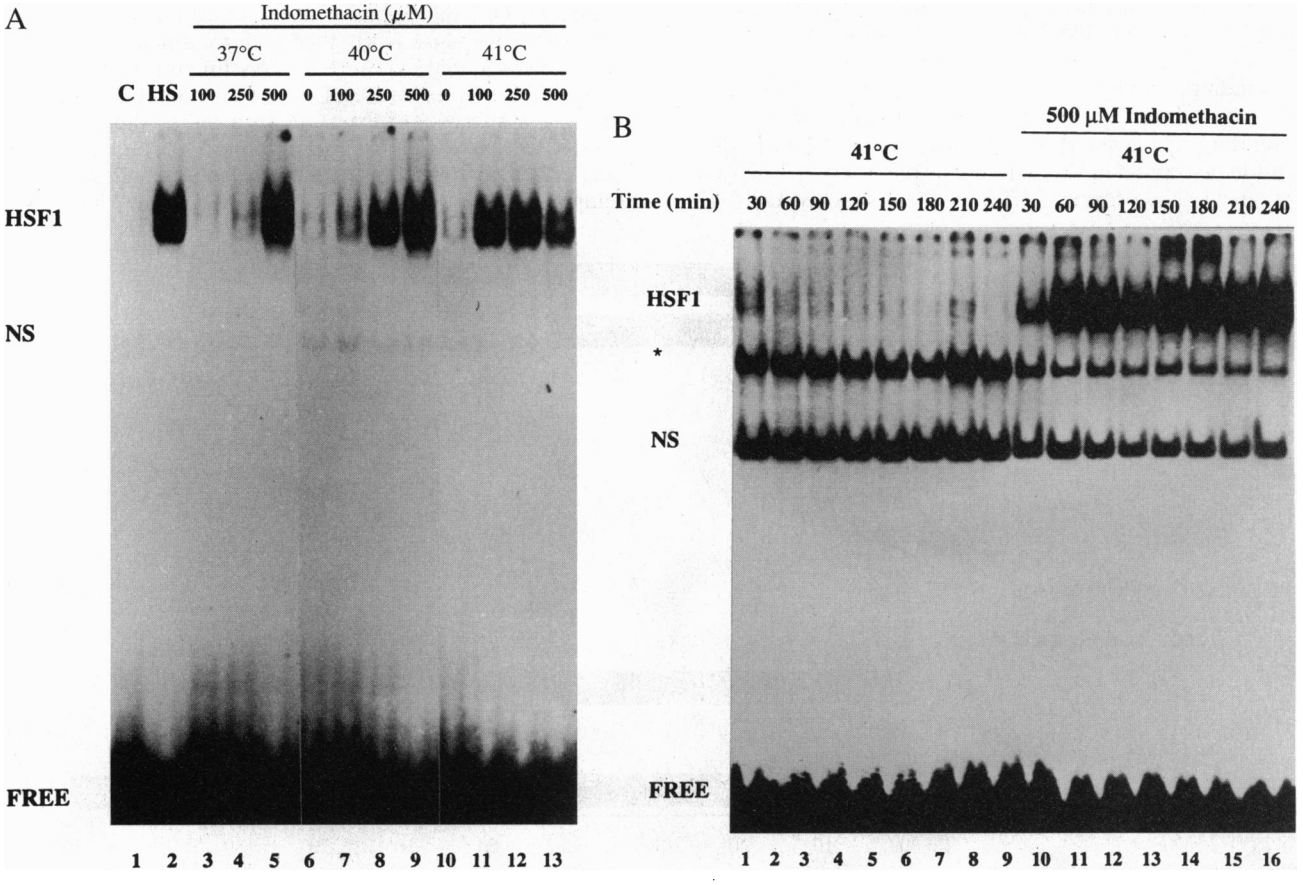

FIG. 3. Indomethacin treatment alters the temperature threshold and activation profile of HSF1 DNA-binding activity. (*A*) Gel mobility-shift analysis of HeLa S3 cells treated with indomethacin, 40°C, or 41°C alone or the combination of indomethacin and either 40°C or 41°C. Cells were treated with 100, 250, or 500 μM indomethacin for 30 min before being exposed to low heat shock temperatures (40°C and 41°C) for an additional 30 min. Cells were also treated with 30 min of 42°C heat shock (HS) or maintained at 37°C (C). NS, nonspecific protein–DNA complex; FREE, unbound heat shock element oligonucleotide. (*B*) Time course of HSF1 DNA binding in HeLa S3 cells treated with either 41°C alone or 500 μM indomethacin and 41°C simultaneously. Whole-cell extracts were prepared and gel mobility-shift analysis was performed. *, Constitutive DNA binding activity; NS, nonspecific complex.

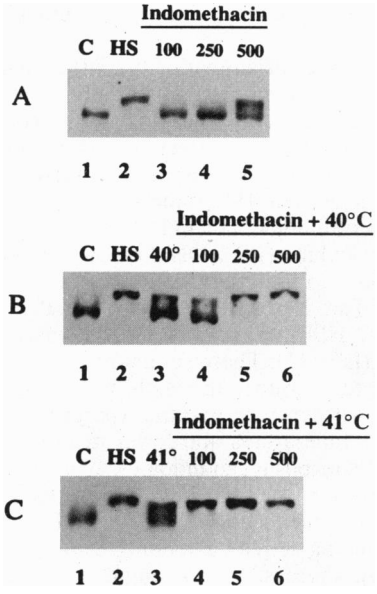

FIG. 4. Modulation of HSF1 phosphorylation by indomethacin and temperature. Western blot analysis of HSF1 from indomethacin-treated, indomethacin + 40°C, and indomethacin + 41°C treated cells. Extracts from treated cells were separated by SDS/PAGE and analyzed by Western blot analysis using a polyclonal antiserum to HSF1. (*A*) Cells exposed to control (C) (37°C) or heat shock (HS) (42°C) temperature or incubated in 100, 250, or 500 μM indomethacin at 37°C. (*B*) Cells exposed to 40°C alone or cotreated with 40°C and 100, 250, or 500 μM indomethacin. (*C*) Cells exposed to 41°C alone or cotreated with 41°C and 100, 250, or 500 μM indomethacin.

DNA-binding activity; however, the indomethacin-induced HSF1 is insufficient to activate heat shock gene transcription. Among the steps in the process of heat shock-induced HSF1 activation is stress-inducible serine phosphorylation. The transcriptionally active form of HSF1 in heat-shocked cells was inducibly phosphorylated, which resulted in slower mobility on SDS/polyacrylamide gels (Fig. 4*A*, lanes 1 and 2). In contrast, HSF1 from the indomethacin-treated samples (Fig. 4*A*, lanes 3–5) comigrated with the control form of HSF1 at low concentrations and was incompletely phosphorylated at 500 μM indomethacin. These results were corroborated by direct ^{32}P-labeling studies and the use of phosphatase treatment, which confirmed that the indomethacin-induced HSF1 was only inducibly phosphorylated at the highest drug concentrations (41). We next examined whether HSF1 was inducibly phosphorylated in cells exposed to the combination of indomethacin and low heat-shock temperatures. Extracts prepared from cells treated simultaneously with suboptimal levels of indomethacin and slightly elevated temperatures were analyzed for the phosphorylation state of HSF1. As shown in Fig. 4 *B* and *C*, HSF1 in cells simultaneously treated with indomethacin and 40°C was completely phosphorylated at 250 μM indomethacin (Fig. 4*B*, lane 5) and partially phosphorylated at 100 μM indomethacin (lane 4). In cells cotreated with 41°C and indomethacin, the phosphorylated form of HSF1 was observed even at the lowest level of indomethacin used (Fig. 4*C*, lane 4). The combination of indomethacin and slightly elevated temperatures resulted in the induced transcription of heat shock genes well above that detected for the same concentrations of drug or temperature alone (data not shown). These results reveal that the effects of synergy extend beyond a reduction of the threshold of HSF1 activation and includes activation of the kinase(s) responsible for inducible HSF1 phosphorylation and stimulation of inducible heat shock gene transcription.

One of the implications of drug-induced modulation of the heat shock response is whether such treatment protects cells against subsequent exposure to stressful conditions. It is well established that exposure of cells to a nonlethal heat shock prior to a subsequent exposure to a lethal shock results in cytoprotection. Therefore, we examined whether indomethacin, either alone or together with suboptimal heat shock temperatures, has a cytoprotective effect against lethal expo-

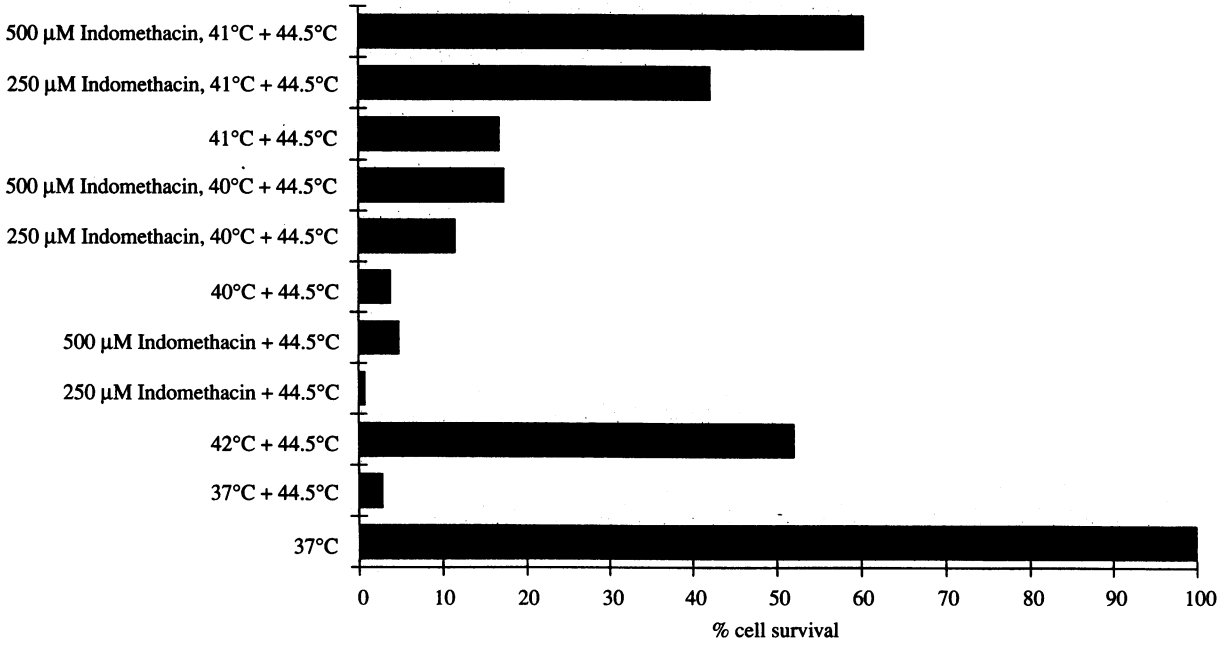

FIG. 5. Synergy between indomethacin and suboptimal heat shock temperatures leads to cytoprotection. HeLa cells were left untreated (37°C); incubated at 44.5°C for 30 min (heat shock) (37°C + 44.5°C); preshocked at 42°C for 1 h, followed by recovery at 37°C for 10 h and heat shock at 44.5°C for 30 min (42°C + 44.5°C); incubated with 250 or 500 μM indomethacin at 37°C prior to being heat shocked at 44.5°C; incubated at either 40°C or 41°C prior to being heat shocked at 44.5°C; or incubated with 250 or 500 μM indomethacin at either 40°C or 41°C before being heat shocked at 44.5°C. After being heat shocked, cells were plated at 37°C, and the number of cells surviving to form colonies was determined after 7 days. All data are normalized to the survival of the control, non-heat-shocked sample, which was set at 100%.

sures to stress. For example, preshock at 42°C, a temperature that induces a maximal heat shock response in HeLa cells, led to substantial cytoprotection (50% survival) to a subsequent 44.5°C challenge, as measured by clonal analysis, whereas exposure to the 44.5°C challenge alone resulted in 3% survival. HeLa cells were incubated with various concentrations of indomethacin, either alone or together with a suboptimal heat shock (40°C/41°C), and subsequently exposed to a normally lethal challenge temperature of 44.5°C. As shown in Fig. 5, exposure of cells to indomethacin, 40°C, or 41°C by itself had a modest effect on cell survival (2–15% survival, respectively). However, indomethacin together with either 40°C or 41°C had a strongly protective effect (12–60% survival), as measured by cell survival and clonal analysis. This cytoprotective effect is closely linked with the activation of HSF1 DNA-binding activity and inducible phosphorylation of HSF1 (Fig. 4).

The observations presented here reveal that indomethacin, as with other antiinflammatory drugs, alters the expression of the human heat shock genes. Although the current studies emphasize the effects of indomethacin, we have examined other NSAIDs, including sodium salicylate, ibuprofin, sulindac, nordihydroguaiaretic acid, and diflunisal, all of which affect HSF1 activity, both directly and in synergy with slightly elevated temperatures (ref. 5; J.C., unpublished data). Here, we demonstrate that the induction of a heat shock response by the synergistic effects of the antiinflammatory drug and elevated temperature may have an important role in protecting cells against stress. Although our studies have been performed on transformed human cells in culture, it is likely that similar results would be obtained with nontransformed primary cells and therefore be relevant *in situ* at the site of inflammation. While indomethacin by itself can directly lead to HSF1 activation, the drug-induced form of HSF1 lacks the properties of a complete transcriptional activator. We have not ruled out the possibility that the transcriptionally inert form of HSF1 is functional, perhaps to act as a repressor of transcription. Indomethacin, accompanied by rises in temperature such as occurs duing inflammation, both lowers the threshold for the induction of the heat shock response and prolongs the period of HSF activation. These observations support an additional role for NSAIDs as cytoprotective agents through their effects on HSF1 activation. Together with our previous demonstration that salicylate and arachidonic acid activate HSF1 (6, 7), these findings strengthen the hypothesis that the events leading to heat shock and inflammatory responses share common pathways. Furthermore, the observations presented here on indomethacin provide strong evidence to support a role for the heat shock response and the biological activities of heat shock proteins and molecular chaperones in the action of NSAIDs. The ability of indomethacin to reduce the temperature threshold of the heat shock response may have direct implications for inflammation and other diseases, as the heat shock response can now be modulated by drugs of clinical importance under physiologically relevant conditions.

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