Antiproliferative prostaglandins activate heat shock transcription factor

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ABSTRACT Treatment of human K562 erythroleukemia cells with the antiproliferative prostaglandin A₃ results in the elevated transcription of two heat shock genes, HSP70 and HSP90. Parallel with increased heat shock gene transcription is the activation of heat shock transcription factor. Heat shock transcription factor levels are induced within 60 min after prostaglandin A₃ addition to levels similar to that achieved during heat shock. The requirement for protein synthesis for prostaglandin A₃ activation of heat shock transcription factor suggests that effects on nascent protein synthesis may be involved in the signaling mechanism. Although it is unclear whether the activation of a heat shock response by prostaglandins is relevant to the biochemical properties of these natural substances, cells pretreated with prostaglandin A₃ are protected against a subsequent heat shock, indicative of a thermotolerant state.

Prostaglandins are a class of naturally occurring cyclic 20-carbon fatty acids that are synthesized from polyunsaturated fatty acid precursors in response to external stimuli such as cell injury and inflammation (1). Prostaglandins function as intracellular hormones involved in the regulation of various physiological and pathological processes of eukaryotes, including cell proliferation and differentiation (2), the immune response (3), inflammation (4), cytoprotection (5, 6), and the febrile response (7).

The type A and J prostaglandins, characterized by the presence of a reactive α,β-unsaturated ketone in the cyclopentane ring (cyclopentenone prostaglandins), have antiproliferative activity and cause cultured mammalian cells to arrest in the G₁ phase of the cell cycle (8–10). Human erythroleukemia K562 cells, for example, are extremely sensitive to prostaglandin A₃ (PGA₃), which results in nearly complete cessation of cell growth at doses that do not affect cell viability and do not suppress DNA or RNA synthesis for at least 24 h (11, 12). Treatment with PGA₂, PGA₃, and PGJ₂ results in the elevated synthesis of HSP70, a major heat shock and stress-induced protein (12, 13). Because HSP70 expression is also growth regulated (14, 15), we reasoned that induction of HSP70 by prostaglandins could represent a response to the growth-related effects of this compound. The growth-regulated response of the human HSP70 gene requires cis-acting elements in the basal promoter that are distinct from the distally located heat shock elements on the heat shock gene promoter, which are necessary for heat shock and other forms of stress responsiveness. The cellular response to a wide range of external stress stimuli, including heat shock, heavy metals, amino acid analogues, oxidizing agents, and teratogens (16), involves the activation of heat shock transcription factor (HSF), which binds to the heat shock element (HSE), comprised of multiple adjacent inverted repeats of the pentamer nGAAAn (17, 18).

In this study, we show that the cyclopentenone prostaglandin PGA₃ induces in human cells the transcription of classical heat shock genes through activation of HSF. The activation of HSF by PGA₃ requires continued protein synthesis, thus suggesting a role for PGA₃ in the modification of nascent proteins.

MATERIALS AND METHODS

Cell Culture. K562 cells were grown at 37°C in RPMI 1640 medium supplemented with 10% (vol/vol) fetal calf serum at densities of 1 x 10⁶ to 1 x 10⁷ cells per ml. Prostaglandins (Cayman Chemicals, Ann Arbor, MI), stored as 100% ethanol stock solution (10 mg/ml), were used at a concentration of 4 μg/ml, unless differently specified, and control cells received a corresponding volume of ethanol.

Transcription, mRNA Levels, Protein Synthesis, and Gel Mobility Shift Assays. In vitro run-on transcription reactions were performed in isolated K562 nuclei as described (19). 3²P-labeled RNA was hybridized to nitrocellulose filters containing plasmids for the following human genes, HSP70 (pH2.3; ref. 20), HSP90 (pUCH8501; ref. 21), HSP60 (pUC601; ref. 22), GRP78/BiP (pH231.1; ref. 23), p72/HSC70 (pHA7.6), β-actin (24), and c-myc (pSV-T; ref. 25), and vector (pGEM2; Promega). Following hybridization, filters were visualized by autoradiography and the radioactivity was quantitated by a Molecular Dynamics Phosphorimager (Molecular Dynamics, Sunnyvale, CA).

Total cytoplasmic RNA was isolated (26), fractionated on 1% agarose/formaldehyde gels, transferred to nitrocellulose, and hybridized with nick-translated 3²P-labeled HSP70 gene sequences.

Cells were metabolically labeled with [³⁵S]methionine for 45 min and equal amounts of protein were analyzed on 10% SDS/PAGE gels. For immunoblot analysis, the nitrocellulose filters were incubated with monoclonal antibody 3A3, which recognizes HSP70 and p72/HSC70 (S. P. Murphy, personal communication). The filters were incubated with horseradish peroxidase-conjugated goat anti-mouse IgG (Boehringer Mannheim), and specific complexes with HSP70 and p72/HSC70 were detected by enhanced chemiluminescence (Amersham).

Whole cell extracts were prepared and binding reactions were performed using a specific heat shock element probe as described (27). HSF–HSE complexes were analyzed by non-denaturing 4% polyacrylamide gel electrophoresis.

RESULTS

PGA₃ Induces Transcription of HSP90 and HSP70. In previous studies it was observed that treatment of cultured human cells with PGA₂, PGE, and PGF resulted in the elevated synthesis of HSP70 only in cells treated with PGA₂. To examine whether PGA₃ exerted its effect at the transcriptional level, we measured the effects of PGA₃ on the tran-

Abbreviations: PG, prostaglandin; HSF, heat shock transcription factor; HSE, heat shock element.
scription of a number of human genes, including the heat shock genes HSP90, HSP70, and HSP60, GRP78/BiP and p72/HSC70, as well as two control (nonstress) genes, β-actin and c-myc. Knowledge of which genes were induced by PGA1 would reveal whether the effects of PGA1 were specific.

To measure the effects of PGA1 on transcriptional activation, we performed in vitro nuclear run-on assays with nuclei isolated from K562 cells at different times during PGA1 treatment. The transcription rate of the HSP70 gene increased rapidly between 45 and 90 min, attained a maximal 15-fold induction within 3 h, and thereafter declined to basal levels by 10 h (Fig. 1). By comparison, heat shock induced HSP70 transcription by 50-fold (Fig. 1A, HS). Transcription of HSP90, though less striking, exhibited similar kinetics to HSP70. In contrast to the relatively rapid induction of HSP70 and HSP90, GRP78/BiP transcription was consistently induced 4-fold only at 6 h of PGA1 treatment. The transcription of another heat shock gene (HSP60), the constitutively expressed p72/HSC70, and the control β-actin and c-myc genes was unaffected. These results clearly demonstrate that PGA1 acts at the transcriptional level and preferentially induces two classical heat shock genes, HSP70 and HSP90.

We examined the effects of PGA1 on HSP70 mRNA levels during PGA1 treatment. Cytoplasmic RNA was isolated at different times following PGA1 treatment and HSP70 mRNA levels were measured by Northern blot analysis (Fig. 2A).

![Fig. 1. Effects of PGA1 treatment on the transcription of control (nonstress) and heat shock genes in K562 cells. (A) Autoradiogram of the relative transcription rates of plasmid vector (pGEM2), HSP70, HSP90, HSP60, GRP78, p72/HSC70, β-actin, and c-myc genes at the indicated times (min, h) during PGA1 treatment or following heat shock (at 42°C) for periods of 20, 45, and 90 min. (B) Quantitative analysis using a Molecular Dynamics Phosphorimage analyzer of HSP70 (●), HSP90 (▲), β-actin (●), and c-myc (●) transcription rates shown in A. The values are expressed as arbitrary units obtained by comparing transcription rates to control levels.](image)

![Fig. 2. Effect of PGA1 treatment on HSP70 mRNA levels and HSP70 protein synthesis and accumulation. (A) Northern blot analysis of cytoplasmic RNA isolated at various times of PGA1 treatment on control (C) or heat-shocked (HS) cells. The band at 2.6 kilobases corresponds to the hybridization with the HSP70 probe and the 1.3-kb band corresponds to the rat glyceroldehyde-phosphate dehydrogenase mRNA used as an internal control for the loading of RNA. (B) Autoradiography of SDS/PAGE analysis of [35S]methionine-labeled proteins in control, heat-shocked (HS), or PGA1-treated K562 cells. The positions of HSP90, HSP70, GRP78, and actin are indicated. (C) Western blot analysis of duplicate samples as indicated in B, incubated with the monoclonal antibody 3A3, which recognizes p72/HSC70 and HSP70 proteins.](image)

HSP70 mRNA levels were elevated slightly at 90 min, with maximal HSP70 mRNA levels detected at 3–6 h. Thus, the increase in HSP70 mRNA levels followed the increase in HSP70 transcription rates (compare Fig. 1A to Fig. 2A). The effects of PGA1 on the overall levels and patterns of protein synthesis, specifically HSP70 synthesis, were examined by pulse-labeling with [35S]methionine and SDS/PAGE. Slightly increased levels of HSP70 synthesis were detected by 90 min of PGA1 treatment, coincident with the initial increase in HSP70 mRNA levels, and reached higher levels of synthesis between 3 and 6 h (Fig. 2B). HSP90 synthesis was induced slightly after 90 min of PGA1 treatment and GRP78/BiP synthesis was elevated only at later time points. Synthesis of other stress-responsive proteins and overall levels of protein synthesis were not affected by PGA1 treatment. The results of Western blot analysis using a monoclonal antibody (3A3) that recognizes p72/HSC70 and HSP70 revealed that HSP70 levels gradually accumulated during PGA1 treatment to a 3-fold higher level by 6–10 h (Fig. 2C). Although the PGA1 induction of HSP70 gene expression was transient, high levels of HSP70 persisted for periods up to 24 hours. The effects of PGA1 appear to be specific to HSP70 since the levels of p72/HSC70 were unaltered.
PGA\textsubscript{1} Treatment Activates HSF. Two lines of evidence suggested that PGA\textsubscript{1} induced \textit{HSP70} transcription through activation of HSF. First, the kinetics of induction were rapid, and second, two heat-shock-induced genes, \textit{HSP70} and \textit{HSP90}, were induced. These results suggest that transcriptional activation is mediated by HSF. To demonstrate whether PGA\textsubscript{1} induced HSF, we used the gel mobility shift assay using a synthetic oligonucleotide containing the consensus HSE binding site from the human \textit{HSP70} promoter. Gel mobility shift assays were performed with whole cell extracts prepared from cells treated with the same dose (4 µg/ml) of PGA\textsubscript{1}, PGE\textsubscript{2}, and PGF\textsubscript{1α}. Only PGA\textsubscript{1} treatment induced the appearance of a HSF–HSE complex, which was indistinguishable from the heat-shock-induced HSF–HSE complex (Fig. 3A). HSF–HSE complex formation was detected within 90 min of PGA\textsubscript{1} treatment, attained maximal levels between 3–6 h, and declined after 10 h. Kinetics of HSF DNA-binding activity induced by PGA\textsubscript{1} and heat shock are presented schematically in Fig. 3B, showing a pattern that closely parallels the induction of \textit{HSP70} transcription (Fig. 1A). It was of interest to note that the level of \textit{HSP70} transcription was significantly lower in PGA\textsubscript{1} versus heat-shocked cells even though the levels of HSF were equivalent in cells subjected to either treatment (compare Fig. 1A to Fig. 3A and B). To further characterize the PGA\textsubscript{1}-induced activation of HSF and heat shock gene transcription, we performed a dose–response experiment in which K562 cells were treated for 3 h with different concentrations of PGA\textsubscript{1}. The HSF DNA-binding activity and the \textit{HSP70} transcription were induced when cells were treated with PGA\textsubscript{1} at concentrations >2 µg/ml (data not shown).

Activation of HSF by PGA\textsubscript{1} Requires Nascent Protein Synthesis. Little is known about the mechanism of action of the cyclopentenone prostaglandins. It has been reported that PGA\textsubscript{1} is rapidly incorporated into cells and binds to nuclear proteins and DNA (28). One possibility is that PGA\textsubscript{1} interacts with, modifies, and alters the conformation of certain proteins, thus activating the heat shock transcriptional response. To test this hypothesis, we examined whether the PGA\textsubscript{1} induction of heat shock gene transcription and HSF activation was dependent on \textit{de novo} protein synthesis. Cells were treated with 100 µg of cycloheximide per ml for 30 min prior to PGA\textsubscript{1} administration. Cells were removed at various times following treatment and assayed for HSF DNA-binding and transcriptional activities. As shown in Fig. 4A and C, cycloheximide pretreatment abolished the ability of PGA\textsubscript{1} to induce \textit{HSP70} and \textit{HSP90} transcription and activation of HSF. These results strongly suggest that \textit{de novo} protein synthesis is necessary for PGA\textsubscript{1}-induced activation of the heat shock response.

The requirement for protein synthesis for activation of HSF during treatment with PGA\textsubscript{1} contrasts with the requirements for protein synthesis in activation of the heat shock response. Cells were pretreated with cycloheximide and subsequently incubated at 37°C, 42°C, 43°C, and 45°C. As shown in Fig. 4B, \textit{de novo} protein synthesis is required for activation of HSF and heat shock gene transcription at 42°C but not at 43°C and 45°C. These results demonstrate that activation of a 42°C heat shock response is dependent on protein synthesis, which suggests that the mechanism of 42°C heat shock involves damage to nascent translation products. In addition, we suggest that heat shock at 43°C or 45°C affects
Fig. 4. Effect of cycloheximide treatment on PGA1 activation of HSF and heat shock gene transcription. (A) K562 cells were untreated (−) or treated (+) for 30 min with cycloheximide (100 µg/ml) prior to PGA1 treatment for periods as indicated. Whole cell extracts were prepared for gel mobility shift assay using the HSE probe. (B) K562 cells were untreated (−) or treated (+) with cycloheximide prior to a 5-min heat shock at 42°C, 43°C, and 45°C. (C) Transcription rates of the HSP70, HSP90, and β-actin genes measured by nuclear run-on assay from the same samples as shown in A and B (HS at 42°C).

nascent and preexisting proteins, thus providing the signal for activation of the heat shock response that is independent of protein synthesis.

Exposure to Prostaglandin Attenuates Subsequent Prostaglandin Treatment or Heat Shock Response. During the continuous exposure of cells to PGA1, the transcriptional induction of HSP70 and activation of HSF represent a transient response. This can be explained by the depletion of PGA1 from the culture medium, reducing the effective concentration, even though PGA1 has been shown to be stable over a 24-h period at 37°C (29). An alternative possibility is that cells continuously exposed to PGA1 become adapted to PGA1, perhaps related to the transient induction of heat shock gene expression. To distinguish between these possibilities, cells were treated for 15 h with PGA1, during which HSP70 transcription rates were induced and attenuated, and then treated with an additional aliquot of PGA1. Cells were withdrawn at different time points during the second PGA1 treatment and the levels of heat shock gene transcription and HSF DNA-binding activity were measured. Cells that received two treatments of PGA1 had a 50% reduction of HSF DNA-binding activity as compared to the levels of HSF achieved in a single PGA1 treatment (data not shown). The reduced levels of HSF DNA-binding activity were, however, insufficient to stimulate HSP70 transcription above basal levels (Fig. 5A). We also examined whether cells treated with PGA1 had an altered sensitivity to heat shock. PGA1 pretreatment resulted in a consistent 20% reduction in the levels of HSF DNA-binding activity, induced by a 42°C heat shock (data not shown), which corresponded to a 60% reduction in HSP70 transcription (Fig. 5B). These results exclude the possibility that the transient heat shock response during PGA1 treatment is due to depletion of PGA1 in the culture medium and suggest that PGA1-treated cells acquire a form of "tolerance" to subsequent exposures to PGA1 or heat shock.

DISCUSSION

The studies presented here demonstrate that PGA1 treatment of human K562 cells has a significant effect on the expression of two major heat shock genes, HSP70 and HSP90. The inducible transcription of both genes by PGA1 is mediated by HSF, which is activated from its control non-DNA-binding state to a transcriptionally active form. Our studies reveal a surprising specificity of the cyclopentenone prostaglandins since only PGA1 induces HSF DNA-binding activity and the closely related PGE and PGF do not induce the heat shock response.

The requirement of protein synthesis for PGA1 induction of HSF activation suggests that PGA1 may affect nascent pro-
covalent interaction between PGAM and sulphydryl groups of cysteine-rich proteins (31, 32). It will be of interest to examine the specificity of PGAM activity in greater detail. Is the activation of HSF by PGAM due to direct effects on protein conformation? If PGAM interacts and modifies nascent proteins, it will be important to establish whether there are preferred or specific substrates. Because we have not been able to in vitro activate HSF by exogenous PGAM using HeLa cell S100 extracts, it seems unlikely that PGAM acts directly on the control non-DNA-binding form of HSF.

Even though these studies reveal a specific effect of the cyclopentenone prostaglandins on gene expression, an important question is whether the activation of a heat shock-like response by PGAM is biologically relevant to some of the known biological activities of PGAM as antiviral agents (23–29, 33–35) and in the antiproliferative response (10, 13, 36). The effects of prostaglandins and hemin on K562 cells share common features. Hemin treatment causes non-terminal erythroid differentiation, the activation of HSF DNA-binding activity, and the induction of HSP70, HSP90, and GRP78/BIP transcription (ref. 37; L.S. and R.I.M., unpublished data). Prostaglandins and hemin, unlike the traditional inducers of the heat shock response (e.g., inhibitors of energy metabolism, amino acid analogues, antineoplastic compounds), are natural substances. Whether the levels of the cyclopentenone prostaglandins achieve a sufficiently high local concentration in vivo to activate HSF and to transcribe the classical heat shock genes in affected cells or tissues has not been examined.

We have found that pretreatment with PGAM, at concentrations that induce HSF DNA-binding activity, diminishes the magnitude of the subsequent stress response. The attenuation of the second exposure to stress, which occurs in PGAM-pretreated cells, may be related to the accumulation of a 2- to 3-fold higher level of HSP70 protein. One explanation is that the induction and accumulation of high levels of HSP70 during the initial exposure to PGAM alters the ability of the treated cell to sense or respond to a subsequent stress stimulus and thus, by direct or indirect means, to regulate the activity of HSF. Consistent with this view, our laboratory has recently shown an interaction between HSP70 and HSF (38).

The role of PGAM in the regulation of the stress response is of particular interest as prostaglandins are a metabolic product of arachidonic acid, a central mediator of the inflammatory response. A possible link between the inflammatory response and the stress response can be suggested from two recent observations. Treatment of HeLa cells with salicylates and indocin, both nonsteroidal antiinflammatory drugs, as well as with arachidonic acid itself induces HSF DNA-binding activity (ref. 39; D. Jurivich, L.S., and R.I.M., unpublished data).

Note Added in Proof. Holbrook et al. (40) have recently made similar observations on PGA effects on the heat shock response.

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