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Heat Shock Factors and the Control of the Stress Response

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ABSTRACT. Living cells are continually challenged by conditions which cause acute and chronic stress. To adapt to environmental changes and survive different types of injuries, eukaryotic cells have evolved networks of different responses which detect and control diverse forms of stress. One of these responses, known as the heat shock response, has attracted a great deal of attention as a universal fundamental mechanism necessary for cell survival under a variety of unfavorable conditions. In mammalian cells, the induction of the heat shock response requires the activation and translocation to the nucleus of one or more heat shock transcription factors which control the expression of a specific set of genes encoding cytoprotective heat shock proteins. The discovery that the heat shock response is turned on under several pathological conditions and contributes to establish a cytoprotective state in a variety of human diseases, including ischemia, inflammation, and infection, has opened new perspectives in medicine and pharmacology, as molecules activating this defense mechanism appear as possible candidates for novel cytoprotective drugs. This article focuses on the regulation and function of the heat shock response in mammalian cells and discusses the molecular mechanisms involved in its activation by stress and bioactive cyclopentenone prostanoids, as well as its interaction with nuclear factor κB, a stress-regulated transcription factor with a pivotal role in inflammation and immunity. BIOCHEM PHARMACOL **59**;1:55–63, 2000.

KEY WORDS. antiviral; cyclopentenone; heat shock factor; heat shock proteins; heat shock response; NF-κB; prostaglandins

THE HEAT SHOCK RESPONSE

The observation that an increase in temperature of a few degrees above the physiological level induces the synthesis of a small number of proteins in *Drosophila* salivary glands led to the discovery of a universal protective mechanism which prokaryotic and eukaryotic cells utilize to preserve cellular function and homeostasis [1]. This complex physiological defense mechanism, known as the heat shock response, involves the rapid induction of a specific set of genes encoding cytoprotective proteins (heat shock proteins, HSP†) [1, 2]. In mammalian cells, HSP synthesis is induced not only by hyperthermia, but can be triggered by a wide variety of toxic conditions which lead to the accumulation of non-native proteins, including alterations in the intracellular redox environment, exposure to heavy metals, amino acid analogs or cytotoxic drugs, glucose deprivation, and virus infection [3]. Activation of the transcription of heat shock genes is also modulated under non-stressful conditions during progression through the cell cycle, during development and differentiation, or following exposure to molecules that regulate cell proliferation [3, 4]. HSP are highly conserved, ubiquitous, and abundant in nearly all subcellular compartments. They are divided into different families, according to molecular size (i.e. hsp100, hsp90, hsp70, hsp60, hsp40, and small HSP). In mammalian cells, several HSP that function as molecular chaperones and are essential for a correct folding, assembly, and intracellular translocation of proteins are expressed during normal growth conditions and can be induced by biologically active molecules such as hemin [4] and prostaglandins [5], whereas others are expressed upon stress-activated regulation of transcriptional and translational switches [1, 2]. While prolonged exposure to conditions of extreme stress is harmful and can lead to cell and tissue death, induction of HSP synthesis can result in stress tolerance and cytoprotection against stress-induced molecular damage [6]; furthermore, transient exposure to elevated temperatures has a cross-protective effect against sustained, normally lethal exposures to other forms of stress. A common protective function of HSP during stress is to hold nonnative proteins as soluble folded intermediates in a refolding competent state, thus functioning as kinetic traps to

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† *Abbreviations:* HSP, heat shock proteins; HSF, heat shock transcription factor, HSE, heat shock element; PGA, A-type prostaglandin; PGJ, J-type prostaglandin; cyPG, cyclopentenone prostaglandin; HIV-1, human immunodeficiency virus type 1; NF-κB, nuclear factor-kappa B; and IKK, IκB kinase.

prevent off-pathway intermediates and formation of aggregates [2, 7].

One of the most studied HSP families is the 70 kDa HSP (hsp70), whose structure has been widely conserved through evolution from bacteria to man, indicating an important role in the survival of the organism [1, 2]. The eukaryotic hsp70 multigene family encodes compartment-specific chaperones, including the constitutively expressed hsc70, the major inducible hsp70, the inducible hsp72, the glucose-regulated grp78/BiP, and the mitochondrial P75, which are localized to the cytosol, nucleus, lumen, and mitochondria, where they maintain proteins in intermediate folded states competent for translocation across membranes and subsequent refolding to the native state [8, 9]. Hsp70 proteins were shown to protect cells against a variety of stresses, including lethal heat shock, anoxia, heavy metals, and arsenite and ethanol toxicity [3, 6]. A cytoprotective role of hsp70 has also been reported in several human diseases, as described below. Moreover, members of the hsp70 family appear to play a direct role in the autoregulation of the heat shock response.

HEAT SHOCK FACTORS

In eukaryotic cells, heat regulation of *hsp* genes requires the activation and translocation to the nucleus of a transregulatory protein, the HSF, which recognizes modular sequence elements referred to as HSE located within the *hsp* gene promoters [10, 11]. The HSE consist of a series of pentameric units arranged as inverted adjacent arrays of the sequence 5′-nGAAn-3′ [12, 13]. A functional HSE is composed of at least three pentamers, and additional reiteration of the pentameric unit results in higher affinity interactions between HSF and HSE [14]. In mammalian hsp70 and hsp90 promoters, the HSE is composed of five and six pentameric units, respectively, in close proximity to basal promoter elements, whose function is independent of the heat shock response [15].

Whereas a single type of HSF has been described in yeast and *Drosophila*, an HSF multigene family has been identified in plants and vertebrates. At least three HSFs (HSF1–3) have been isolated from the human, mouse, and chicken genomes, while an additional factor, HSF4, has been described in human cells [16–20]. Interspecies comparisons indicate 85–95% conservation in amino acid sequences for vertebrate HSF1, whereas, within a single species, the HSFs are approximately 40% related in their sequences. HSFs from different organisms share a number of structural features, including a conserved DNA-binding domain (approximately 100 amino acids), which exhibits a winged helix-turn-helix motif, located near the amino terminus [21]. Adjacent to the DNA-binding domain is a second conserved region that contains three leucine zipper repeats responsible for trimerization of the factor [22]. In *Drosophila* and in larger eukaryotes with the exception of human HSF4, the carboxy terminus contains an additional leucine zipper which has been suggested to have a role in the negative regulation of HSF activity, suppressing trimer formation [19, 23]. Transcriptional activation domains have been mapped to regions near the carboxy terminus of HSFs [24].

In mammalian cells, HSFs are co-expressed, negatively regulated, and activated upon specific environmental and physiological events [11, 25]. HSFs 1 and 3 function as stress-responsive activators and both are required for maximal heat shock responsiveness [26], whereas HSF2 is activated during embryonic development and differentiation [4, 18]. HSF4 was discovered in human cells and appears to be preferentially expressed in the human heart, brain, skeletal muscle, and pancreas [20]. Differently from the other HSFs, HSF4 constitutively binds to DNA, but lacks the properties of a transcriptional activator, and it has been suggested to be a negative regulator of the heat shock response [20]. The presence of different HSFs in larger eukaryotes suggests that an interplay among these factors may be important to protect complex organisms which are exposed to diverse forms of developmental and environmental changes.

REGULATION OF THE HEAT SHOCK RESPONSE

Molecular chaperones such as members of the hsp70 family appear to play a direct role in the autoregulation of the heat shock response. In larger eukaryotes, HSF1 is present in both unstressed and stressed cells. However, in the absence of stress, HSF1 is expressed as an inert monomer bound to hsp70 and other chaperones, and as lacking in transcriptional activity [11, 27]. Both the DNA-binding activity and the transcriptional transactivation domain are repressed through intramolecular interactions and constitutive serine phosphorylation [11, 25].

How do eukaryotic cells sense a change in environmental temperature and activate HSF1? It is commonly held that the stress signal is the consequence of the flux of non-native proteins which, in turn, results in the cellular requirement for molecular chaperones, including hsp70, hsp90, and the co-chaperone Hdj1, to prevent the appearance and aggregation of misfolded proteins (Fig. 1). Chaperones bound to HSF1 would then be sequestered by cellular damaged proteins. As a consequence of the appearance of non-native proteins and release of interacting chaperones, HSF1 DNA-binding activity is de-repressed and monomers oligomerize to a trimeric state, translocate to the nucleus where they become inducibly phosphorylated at serine residues, and bind to HSE located upstream of *hsp* genes, resulting in stress-induced transcription [11, 25]. Inducible phosphorylation appears to be essential for transcriptional activation. For example, chemicals such as salicylates and the nonsteroidal anti-inflammatory drugs (NSAIDs) aspirin and indomethacin cause HSF1 trimerization, nuclear translocation, and binding to the HSE of the endogenous *hsp70* gene; however, they are unable to trigger HSF1 phosphorylation, thus inducing a transcrip-

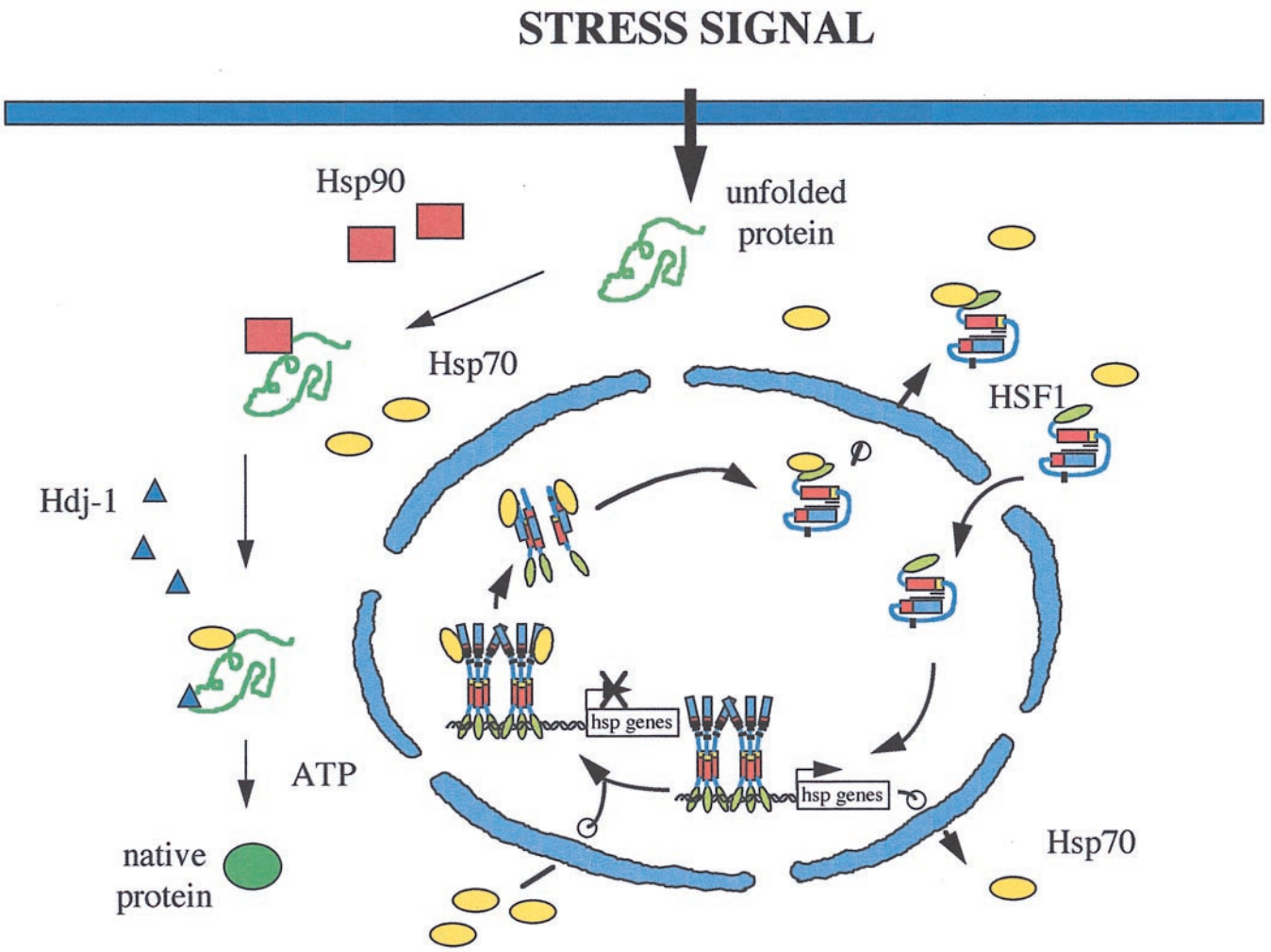

FIG. 1. Regulation of the heat shock response. In unstressed cells, HSF1 is found in the cytoplasm as an inert monomer (shown as intramolecularly negatively regulated for DNA-binding activity), bound to Hsp70 and other chaperones. Activation of HSF1 is linked to the appearance of non-native proteins and the requirement for molecular chaperones (Hsp90, Hsp70, and Hdj1) to prevent the appearance of misfolded polypeptides. As a consequence of the release of interacting chaperones, HSF1 monomers translocate to the nucleus, oligomerize to a trimeric state, become inducibly phosphorylated at serine residues, and bind to HSE located upstream of *hsp* genes, resulting in transcriptional activation and synthesis of heat shock proteins (Hsp70). As the synthesis of HSP increases, Hsp70 and other chaperones relocalize to the nucleus and bind to HSF1, thereby repressing *hsp* gene transcription, and leading to dissociation of trimers and refolding of HSF1 to the inert monomeric state.

tionally inert DNA-binding trimeric state, where expression of *hsp* genes is not detected [28–30]. On the other hand, salicylate- or NSAID-treated cells are primed for subsequent exposure to heat shock and other stresses, leading to the enhanced transcription of heat shock genes [28, 29]. Moreover, alterations of HSF1 phosphorylation by exposure to the calcium ionophore A23187 lead to inhibition of *hsp* gene expression [31]. Whereas inducible phosphorylation is believed to be important for transcriptional activation [11], the kinase (or kinases) involved are still unknown. The identification of the signaling pathway controlling this activity would be a major advance in the understanding of the regulation of the heat shock response in mammalian cells.

As the synthesis of HSP increases to levels proportional to the appearance of non-native proteins, hsp70 and other chaperones relocalize to the nucleus and bind to the HSF1 transcriptional transactivation domain, thereby repressing transcription of heat shock genes [2, 27]. Attenuation of the heat shock response is also dependent on the negative regulatory effects of heat shock factor binding protein 1 (HSBP1), which binds to the region of HSF1 corresponding to the heptad repeat, leading to dissociation of trimers and refolding to the inert monomeric state, thus completing the cycle [32].

Whereas HSF1 is considered the rapidly activated stress-responsive factor, the co-expressed HSF2 is activated in response to distinct developmental cues or differentiation stimuli. HSF2 was shown to be converted from an inert dimer to an active trimer during hemin-induced erythroid differentiation in K562 human erythroleukemia cells [4]. Differently from the rapid activation and attenuation of

HSF1, HSF2 requires a period of 16–24 hr to be activated and remains in the trimeric activated state through 72 hr. Like HSF2, chicken HSF3 is also found as an inert dimer; however, HSF3 shares many characteristics with HSF1, such as negative regulation, activation to trimer, and sequence-specific binding to HSE [19]. HSF3 is activated mainly upon exposure to extreme temperatures and under conditions of severe stress, and its kinetics of activation exhibits a delayed response as compared to HSF1 [19, 26]. As anticipated above, HSF3 appears to be an important co-regulator of HSF1, enhancing the cellular ability to tightly regulate the heat shock response [26]. HSF4, which lacks the leucine zipper in the carboxy terminus portion of the protein, was shown to constitutively bind to DNA, but to be unable to activate transcription [20]. The fact that transient transfection of HSF4 in HeLa cells, which do not express this factor, results in a reduction of HSP synthesis has suggested that HSF4 is a negative regulator of the heat shock response, whose function is to repress the expression of *hsp* genes [20].

CYTOPROTECTIVE ROLE OF HSP IN DISEASE

In the last few years, increased expression of HSP has been described in association with a variety of diseases, including ischemia and reperfusion damage, fever and inflammation, metabolic disorders, cell and tissue trauma, aging, infection, and cancer [2, 3]. Does HSP expression just reflect a state of cellular damage or does it represent an adaptation for survival in a specific pathophysiological state? Whereas HSP induction was at first interpreted as a signal for detection of physiological stress, it is now established that HSP are utilized by the cells in the repair process following different types of injury to prevent damage resulting from the accumulation and aggregation of non-native proteins [2, 3].

The cytoprotective role of HSP, and in particular of hsp70, has been extensively documented *in vitro* as well as *in vivo* in a variety of human diseases, including metabolic disorders [33], inflammation [34], infection [35], and ischemia [36]. In cardiac tissue, different types of insults, including myocardial ischemia, trauma, and hyperthermia, result in the synthesis of HSP, which have been shown to play a pivotal role in restoring normal cardiac function after injury *in vitro* as well as in animal models [36, 37]. Transfected heart-derived cells overexpressing hsp70 and hsp70-transgenic mice show enhanced resistance to ischemic stress, providing evidence for a direct role of this protein in cytoprotection after this type of injury [36, 38]. Moreover, the hsp70 inducer herbimycin A was found to protect rat neonatal cardiomyocytes by simulated ischemia [39]. A pharmacological approach to hsp70 induction and cardiac protection is also suggested by the cytoprotective activity during ischemia of a hydroxylamine derivative currently in phase II clinical trials [40]. In the case of inflammation, HSP protect mammalian cells from tumor necrosis factor α- and β-mediated cytotoxicity [41], and were shown to suppress astroglial-inducible nitric oxide synthase expression [42]. In a rodent model for adult respiratory distress syndrome (ARDS), heat shock-induced hsp70 accumulation within the lung has been associated with decreased pulmonary inflammation and prevention of lethality [43]. Increased expression of hsp70 has also been associated with inhibition of virus replication during acute infection [35, 44]. These observations suggest new therapeutic strategies relying upon the development of drugs that selectively turn on heat shock genes [2]. The understanding of the molecular mechanisms that regulate the heat shock response in mammalian cells has identified the activation of HSF1 as a desirable target for novel cytoprotective drugs.

CYCLOPENTENONE PROSTANOIDS AND THE STRESS RESPONSE

We have shown that cyclooxygenase cyclopentenone metabolites such as prostaglandins of the A and J type (PGAs and PGJs) induce the synthesis of hsp70 in a non-stressful situation in a wide variety of human and mammalian cells [5, 45]. Hsp70 induction is mediated by cycloheximide-sensitive activation of HSF1, which translocates to the nucleus and is kept in a DNA-binding phosphorylated state for several hours upon treatment with cyclopentenone prostaglandins (cyPG) (Fig. 2A) [45, 46]. Consequently, transcription and translation of hsp70 is sustained for long periods of time (12–24 hr, depending on the type of prostaglandin) in human cells. The PGJ metabolite 15-deoxy-$\Delta^{12,14}$-PGJ_2, which was shown to be the natural ligand for the nuclear receptor PPAR-γ peroxisome proliferator-activated receptor-γ, involved in adipocyte differentiation [47], is also a potent inducer of HSF1 activation [48]. PGA_1 and PGJ_2 also induce the synthesis of the oxidative stress-regulated proteins heme oxygenase and ferritin in human monocytes from healthy donors [49]. Structure–activity relationship studies have shown that HSP induction requires the presence of a reactive α,β-unsaturated carbonyl group in the cyclopentane ring (cyclopentenone), which renders this portion of the molecule able to form Michael adducts with cellular nucleophilics, and to covalently bind to cysteine residues of proteins [50]. We have identified the component of the PGA_1 molecule that is responsible for activating HSF1 and triggering rapid and selective transcription and translation of the *hsp70* gene; this small molecule, the 2-cyclopenten-1-one, leads to the accumulation of high levels of hsp70 protein in human cells [51].

Induction of hsp70 by cyPG is associated with a cytoprotective effect during hyperthermia and virus infection. cyPG were shown to induce a thermotolerant state in human cells and to protect cells from subsequent lethal injury [52]. Cyclopentenone prostanoids also exhibit anti-inflammatory [53] and antiviral activity against several viruses, including HIV-1 [44, 54, 55]. Similarly to interferon, cyPG act at multiple levels during the virus replica-

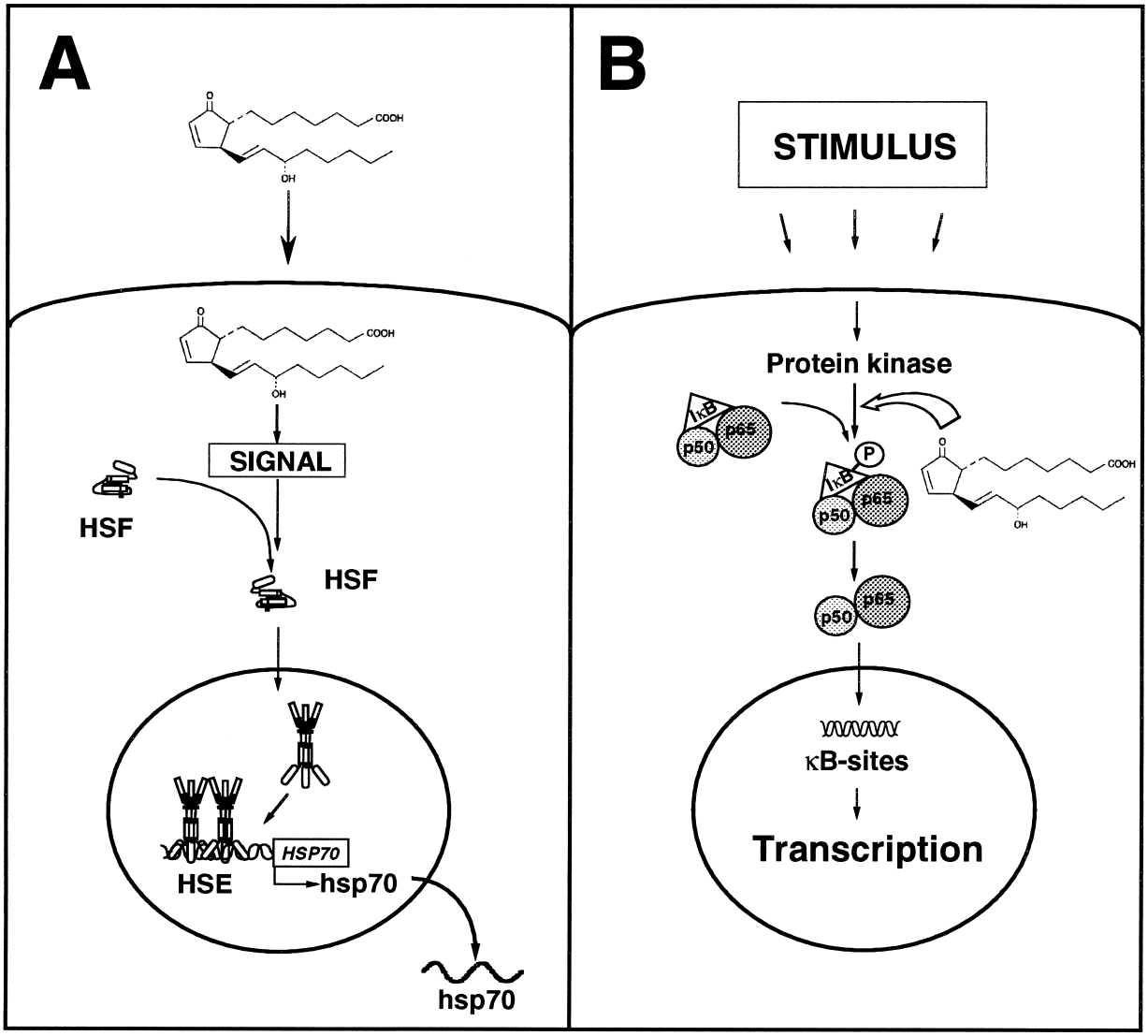

FIG. 2. Activation of the heat shock response and inhibition of NF-κB by cyPG. (A) Synthesis of the cytoprotective 70 kDa HSP (hsp70) is induced by PGA_1 via the activation of HSF1. HSF1 converts from a monomeric non-DNA-binding form to an oligomeric form that translocates to the nucleus and binds to HSE located upstream of the *HSP70* gene. (B) Inhibition of NF-κB activation. NF-κB normally exists in an inactive cytoplasmic complex whose predominant form is a heterodimer composed of p50 and p65 subunits, bound to the inhibitory protein IκBα. cyPG act by blocking IκBα phosphorylation and degradation. Inhibition of NF-κB is associated with HSF1 activation.

tion cycle. In the case of negative-strand RNA viruses, cyclopentenone PGs provoke a selective and dramatic block of virus protein synthesis [35, 56]. This block is exerted at the translational level and is dependent on hsp70 expression in infected cells [35, 44]. In the case of retrovirus infection, a single treatment with PGA_1 or PGJ_2 is cytoprotective in human lymphoblastoid cells acutely infected with HIV-1, and causes an over 1000-fold reduction in infectious virus yield, due to a selective block of HIV-1 mRNA transcription and translation [57, 58]. As HIV-1 mRNA transcription is controlled by the cellular stress-regulated transcription factor NF-κB [59], these observations led us to investigate the possibility that cyPG could also interfere with other stress-regulated pathways.

NF-κB AND THE HEAT SHOCK RESPONSE

NF-κB, an inducible eukaryotic transcription factor of the *rel* family, is a critical regulator of the immediate early pathogen response and activation of the immune system [60, 61]. It normally exists in an inactive cytoplasmic complex, whose predominant form is a heterodimer composed of p50 and p65 (Rel A) subunits, bound to inhibitory proteins of the IκB family, usually IκBα, and is activated in response to a variety of pathogenic stimuli, including viral and bacterial infection, and exposure to UV radiation or to inflammatory cytokines [60, 61]. Upon stimulation, IκBα is phosphorylated by the IKK complex, which contains two catalytic subunits (IKK-α and IKK-β) and the regulatory

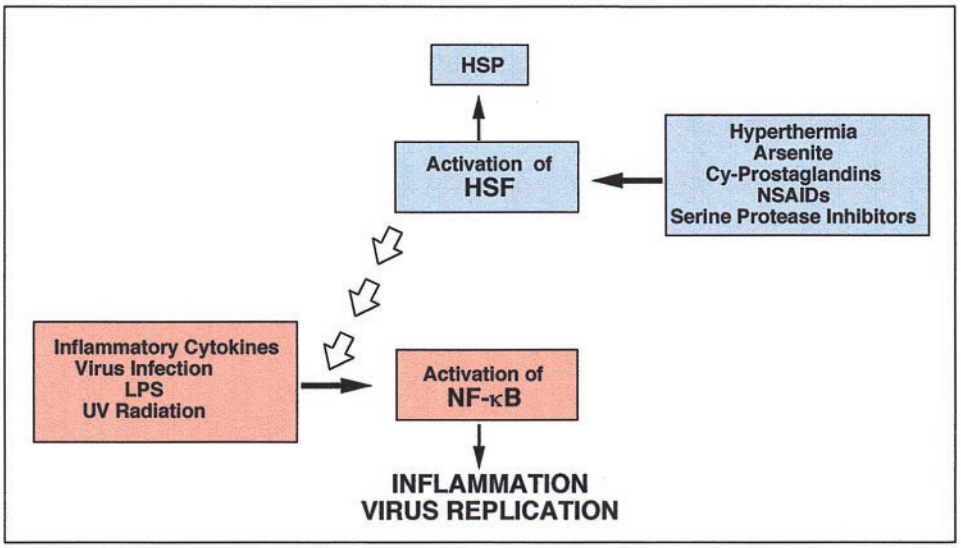

FIG. 3. The relationship between the heat shock response and the activation of NF-κB. Activation of HSF1 by a variety of inducers of the heat shock response, including hyperthermia, sodium arsenite, cyPG, nonsteroidal anti-inflammatory drugs (NSAIDs), and serine protease inhibitors results in inhibition of the activation of NF-κB by different types of stimuli, leading to the simultaneous activation of cytoprotective genes and down-regulation of inflammatory and viral genes. LPS, lipopolysaccharides.

subunit IKK-γ, at sites that trigger its ubiquitination and degradation via the proteasome [62–64]. Release of IκBα results in translocation of NF-κB dimers to the nucleus, where the factor binds to DNA at specific κB sites, rapidly inducing a variety of genes encoding signaling proteins. Target genes include cyclooxygenase-2, inducible nitric oxide synthase, several inflammatory and chemotactic cytokines, cytokine receptors, cell adhesion molecules, as well as viral genes [60, 61].

NF-κB is involved in many pathological events, including inflammation and the progression of AIDS by enhancing transcription of HIV-1 [59]. Consequently, NF-κB is an attractive therapeutic target for novel anti-inflammatory and antiviral drugs, and the need for the development of effective NF-κB inhibitors with therapeutic efficacy is widely recognized. Known inhibitors of NF-κB include serine protease inhibitors [65], caffeic acid phenethyl ester [66], curcumin [67], as well as corticosteroids [68] and nonsteroidal anti-inflammatory drugs including aspirin, though these last compounds act only at very high concentrations [69]. We have shown that cyPG are potent inhibitors of NF-κB activation by cytokines, phorbol esters, or virus infection in human cells [48]. These eicosanoids act by inhibiting the phosphorylation and preventing the degradation of the NF-κB inhibitor IκBα (Fig. 2B) [48]. IKK has recently been identified as the molecular target for cyPG, which were found to directly bind to a cysteine residue in the activation loop of the IKKβ subunit.* Inhibition is dependent on the presence of a reactive cyclopentenone moiety, and results in the blocking of NF-κB-dependent HIV-1 transcription in transient transfection experiments [48].

Both the induction of hsp70 and inhibition of NF-κB appear to contribute to the antiviral activity of cyPG [44]. Most interestingly, these two events appear to be correlated, as suggested by several observations. Inhibition of NF-κB by cyclopentenone prostanoids has been shown to be always associated with and temporally correlated to activation of HSF1 [48]. Also, inhibition of NF-κB by cyPG can be mimicked by other inducers of the heat shock response, including chemical inducers such as sodium arsenite [48], nonsteroidal anti-inflammatory drugs [69], the heavy metals cadmium and zinc,† or by hyperthermia itself [48]. Moreover, a class of well-known NF-κB inhibitors, the serine protease inhibitors including 3,4-dichloroisocoumarin, were recently found to induce hsp70 synthesis via HSF1 activation and to possess antiviral activity [65]. In this case as well, the ability of these molecules to inhibit NF-κB was shown to be closely associated with activation of HSF1 [65]. These results indicate a link between the regulatory pathways of these factors and suggest the possibility that triggering of HSF1 could render mammalian cells unresponsive to stimulation of NF-κB (Fig. 3). If this is the case, the heat shock response could regulate other aspects of the cellular stress response, and inhibition of NF-κB could contribute to the well-known anti-inflammatory and cytoprotective effects that follow the activation of the heat shock response in human cells.

The mechanism by which the heat shock response interferes with the activation of NF-κB is not known. Data from our laboratory have shown that, apart from cyPG, different chemically unrelated inducers of the heat shock response all protect IκBα from stress-induced degradation.‡

* Rossi A, Kapahi P, Natoli G, Takahashi T, Chen Y, Karin M and Santoro MG, manuscript submitted for publication.

† Belardo G, Amici C and Santoro MG, manuscript submitted for publication.

‡ Amici C, Belardo G, Rossi A and Santoro MG, unpublished results.

It could be hypothesized that, due to their chaperone function, one or more HSP could bind to either the NF-κB subunits, to one of the kinases involved in IκBα phosphorylation, or to IκBα itself, thus blocking its proteasome-dependent degradation. Alternatively, an early event in the signaling cascade that leads to HSF1 activation could interfere with IκBα phosphorylation, implying the existence of a cross-talk between the kinases involved. It should be emphasized that, even though results from several studies suggest this possibility, direct evidence that the heat shock response is actively involved in the control of NF-κB is still lacking, and we still have little understanding of how different networks of stress-regulated responses interact with each other.

CONCLUSION

The pharmacological manipulation of endogenous cellular defense mechanisms such as the heat shock response represents an innovative approach to therapeutic intervention in diseases that cause tissue damage. The identification of the molecular structure of prostaglandins responsible for HSF1 activation, triggering rapid and selective expression of the *hsp70* gene in human cells [51], opens new perspectives for the design and development of a class of cytoprotective molecules devoid of the pleiotropic effects of natural eicosanoids. Finally, the possibility of an interaction between the transcription factors HSF1 and NF-κB, which play opposite roles in cytoprotection and cell injury, suggests potential novel therapeutic strategies relying upon the simultaneous activation of cytoprotective genes and down-regulation of inflammatory and viral genes.

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