2-Cyclopenten-1-one, a New Inducer of Heat Shock Protein 70 with Antiviral Activity*

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The cytoprotective role of heat shock proteins (HSP) described in variety of human diseases, including ischemia, inflammation, and infection, suggests new therapeutic strategies relying upon the development of drugs that selectively turn on heat shock genes. Cyclopentenone prostaglandins, which contain an α,β-unsaturated carbonyl group in the cyclopentane ring and possess antiviral activity against several RNA and DNA viruses, were shown to function as signal for HSP synthesis in a nonstressful situation in a variety of mammalian cells. We now report that 2-cyclopenten-1-one selectively induces the expression of the 70-kDa HSP (HSP70) in human cells, through cycloheximide-sensitive activation of heat shock transcription factor 1 (HSF1). The α,β-unsaturated carbonyl group is the key structure triggering HSF1 activation. Induction is associated with antiviral activity during infection with vesicular stomatitis virus. These results identify the molecular structure of natural prostaglandins responsible for HSF1 activation and open new perspectives in the search for novel antiviral and cytoprotective drugs.

Heat shock proteins (HSP) and molecular chaperones have been known for several years to protect cells against a wide variety of toxic conditions, including extreme temperatures, oxidative stress, virus infection, and the exposure to heavy metals or cytotoxic drugs (1). HSP induction is not only a signal for detection of physiological stress, but is utilized by the cells in the repair process following different types of injury, to prevent damage resulting from the accumulation of non-native proteins (2). In mammalian cells, several HSP are expressed during normal growth conditions and can be induced by biologically active molecules such as hemin (3) and prostaglandins (4, 5), whereas others are expressed upon stress-activated regulation of transcriptional and translational switches. Induction requires the activation and translocation to the nucleus of a transregulatory protein, the heat shock transcription factor (HSF), which converts from a monomeric non-DNA-binding form to an oligomeric form that binds to specific promoter elements (HSE) located upstream of heat shock genes (6). Several HSPs have been identified in mammalian cells (6).

We have shown previously that prostaglandins of the A and J type (PGAs and PGJs) were able to activate HSF and induce the synthesis of the 70-kDa HSP (HSP70) in a wide variety of mammalian cells (4, 7). Induction of HSP70 by prostaglandins 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's adducts with cellular nucleophiles and to covalently bind to cysteine residues of proteins via a thioether bond (8, 9). We have also shown that cyclopentenone prostaglandins possess a potent antiviral activity against RNA and DNA viruses in a wide variety of experimental models in vitro and in vivo (reviewed in Refs. 7 and 10). In negative-strand RNA viruses the antiviral activity has been associated with the ability of cyclopentenone prostaglandins to induce HSP70 expression, and a role of this protein in the control of virus replication has been hypothesized (10, 11). We now report that the cyclopentenone ring structure itself, 2-cyclopenten-1-one, is able to activate HSF and selectively induce HSP70 expression and show that the presence of an α,β-unsaturated carbonyl group is the key structure for triggering HSF activation. We also show that induction of HSP70 is associated with inhibition of infectious virus production and viral protein synthesis in monkey kidney epithelial cells infected with vesicular stomatitis virus (VSV).

EXPERIMENTAL PROCEDURES

Cell Culture and Treatment—K562 human erythroleukemia cells and epithelial monkey kidney cells (MA104 cell line) were grown in RPMI 1640 medium, supplemented with 10% fetal calf serum and antibiotics at 37°C in a 5% CO2 humidified atmosphere. For heat shock treatment, flasks were submerged in a temperature-controlled water bath (Grant Instruments, Cambridge, United Kingdom) at 45°C ± 0.01°C for 20 min (immersion depth, 4 cm; t0.5 = 1.5 min). 2-Cyclopenten-1-one (Aldrich), cyclopentanone, cyclopentene, 1-oxo-3,ol, and oenanthic acid (Fluka Chemie AG, Buchs, Switzerland) were dissolved in dimethyl sulfoxide, and control medium contained the same concentration of dimethyl sulfoxide diluent (0.1%). Prostaglandin A1 (Cayman Chemical Co., Ann Arbor, MI) was dissolved in absolute ethanol (10 mg/ml) and diluted to the appropriate concentration in culture medium immediately before use.

Virus Infection and Titration—Confluent MA104 monolayers were washed with phosphate-buffered saline (PBS) and infected with VSV, Indiana serotype (Orsay) (12), 1 plaque-forming unit/cell. After incubation for 1 h at 37°C, virus inocula were removed, and monolayers were washed three times with PBS and incubated with 1 ml of RPMI 1640 containing 2% fetal calf serum and different concentrations of 2-cyclopenten-1-one. Control media contained the same concentration of dimethyl sulfoxide diluent, which was shown not to affect cell or virus metabolism. For VSV titration, medium was collected 24 h post-infection, and after removing cell debris by centrifugation at 2000 rpm for 15 min, titers were determined by cytopathic effect 50% (CPE 50%) assay on confluent monolayers of MA104 cells in 96-well tissue culture dishes (six dilutions for each sample, eight wells for each dilution) as described previously (12). The dilution that gives 50% cytopathic effect was determined by the interpolating procedure of Reed and Muench (13).

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Electrophoretic Mobility Shift Assay (EMSA)—Whole cell extracts (106 K562 cells) were prepared, and binding reactions were performed using a specific heat shock element oligonucleotide, as described (14). Briefly, extracts (10 μg/sample) were mixed with 0.1 ng of a 32P-labeled HSE oligonucleotide and 0.5 μg of poly(dI-dC) (Pharmacia Biotech Inc.) in 25 μl of binding buffer (10 mM Tris-Cl, pH 7.8, 50 mM NaCl, 1 mM EDTA, 0.5 mM dithiothreitol, 5% glycerol). After 20-min incubation at room temperature, HSF-HSE complexes were analyzed by nondenaturing 4% polyacrylamide gel electrophoresis and autoradiography as described in Ref. 5. The amount of shifted HSE probe was quantitated by Molecular Dynamics PhosphorImager analysis. To determine the specificity of HSF1-DNA binding complexes, whole-cell extracts from K562 cells untreated or treated with 500 μM 2-cyclopenten-1-one were preincubated with different dilutions of anti-HSF1 or anti-HSF2 polyclonal antibodies (kindly provided by R. I. Morimoto, Northwestern University, Evanston, IL) for 15 min before EMSA.

Transcriptional Run-on Assay—In vitro run-on transcription reactions were performed in isolated K562 nuclei as described (15). 32P-labeled RNA was hybridized with nitrocellulose filters containing plasmids for the following human genes: HSF2 (pH2.3) (16), GRP78/BiP (glucose-regulated protein 78) (pHO23.1) (5), HSC70 (heat shock cognate 70) (pHA7.6) (5), HO (heme oxygenase) (HO clone 2/10) (17), and gapdh (rat glyceraldehyde phosphate dehydrogenase) (17), as a control. 32P-labeled RNA was hybridized with nitrocellulose filters containing plasmids for the following human genes: HSP70 (pHA7.6) (5), GRP78/BiP (glucose-regulated protein 78) (pHO23.1) (5), HSC70 (heat shock cognate 70) (pHA7.6) (5), HO (heme oxygenase) (HO clone 2/10) (17), and gapdh (rat glyceraldehyde phosphate dehydrogenase) (17), as a control. Following hybridization, filters were visualized by autoradiography, and the radioactivity was quantitated by a Molecular Dynamics PhosphorImager analyzer.

Protein Synthesis, Immunoprecipitation, and Western Blot Analysis—K562 cells were pulse-labeled with 15S-methionine (100 μCi/107 cells) for 1 h at different times after 2-cyclopenten-1-one addition (4). Cells were washed with PBS and lysed in 400 μl of lysis buffer (20 mM Tris-Cl, pH 7.4, 0.1 M NaCl, 5 mM MgCl2, 1% Nonidet P-40, 0.5% SDS) containing protease inhibitors. After cell lysis, the radioactivity incorporated into trichloroacetic acid-insoluble material was determined, and an aliquot of samples containing the same amount of radioactivity was separated by SDS-PAGE in a vertical slab gel apparatus (3%) and run on electrophoresis. For immunoblot analysis, aliquots of samples containing the same amount of radioactivity were subjected to immunoprecipitation for 2 h at 4°C with monoclonal anti-HSF antibodies (Amersham Corp.). Immunoprecipitates were collected with protein G-Sepharose (Sigma), and washed twice with BW buffer (10 mM sodium phosphate, pH 7.6, 0.1 M NaCl, 1 mM EDTA, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS) and once with BW buffer containing 1 M NaCl. After boiling in sample buffer, immunoprecipitated proteins were separated by SDS-PAGE, as described above. For immunoblot analysis, equal amounts of protein for each sample were separated by SDS-PAGE, transferred to nitrocellulose, and filters were incubated with mouse monoclonal anti-HSF70 antibodies, as described (4).

Statistical Analysis—Statistical analysis was performed using the Student’s t test for unpaired data. Data were expressed as the mean S.E. and p values < 0.05 were considered significant.

RESULTS AND DISCUSSION

Human K562 erythroleukemic cells were exposed to varying concentrations of 2-cyclopenten-1-one or were subjected to heat shock. Whole-cell extracts were analyzed by EMSA to determine HSF activation. 2-Cyclopenten-1-one-induced HSF DNA binding activity in a dose-dependent manner (Fig. 1A). Activation comparable with severe heat shock was achieved with concentrations of 250–500 μM 2-cyclopenten-1-one. As in the case of heat shock, HSF type 1 (HSF1) is the primary component of the cyclopentenone-induced HSE binding activity, as determined by EMSA after preincubation of whole-cell extracts with polyclonal anti-HSF1 or anti-HSF2 antibodies (Fig. 1B).

Heat shock-induced HSF activation is known to be dependent on de novo protein synthesis in the case of a mild (42°C) heat treatment, while HSF activation after severe (45°C) heat shock is independent of cellular protein synthesis (18). In order to determine whether HSF1 activation by 2-cyclopenten-1-one is dependent on de novo protein synthesis, K562 cells were either treated with 500 μM 2-cyclopenten-1-one or were stressed at 42 or 45°C for 20 min, in the presence or in the absence of 100 μM cycloheximide (CHX). Ninety or 180 min after 2-cyclopenten-1-one treatment, or 30 min after heat shock, whole-cell extracts were prepared and subjected to EMSA. As shown in Fig. 1C, 2-cyclopenten-1-one-induced HSF1 activation was found to be dependent on de novo protein synthesis, since it was inhibited by cycloheximide treatment.

To investigate the kinetics of HSF activation by 2-cyclopenten-1-one, whole-cell extracts prepared at different times after treatment with 500 μM 2-cyclopenten-1-one or 3 h after heat shock (45°C for 20 min) were subjected to EMSA. The levels of HSF DNA binding activity in 2-cyclopenten-1-one-treated cells were quantitated by Molecular Dynamics PhosphorImager analysis. As shown in Fig. 2A, activation of HSF by 2-cyclopenten-1-one was detected 1.5 h after treatment, and maximal levels of HSF-HSE complexes were detected 9 h after addition of the drug. In the same experiment, the effect of 2-cyclopenten-1-one on HSP70 mRNA transcription was examined by nuclear run-on analysis. 2-Cyclopenten-1-one specifically induced HSP70 mRNA transcription, while it did not affect the transcription rates of messengers for other stress proteins, including HSC70, the glucose-regulated GRP78/BiP, or heme oxygenase (Fig. 2B). HSP70 mRNA transcription started as soon as 1.5 h after 2-cyclopenten-1-one addition, and transcription rates were maximal by 6–9 h, to decline 24 h after treatment (Fig. 2B).
Induction of HSP70 by 2-Cyclopenten-1-one

**Fig. 2. Effect of 2-cyclopenten-1-one treatment on HSF activation, heat shock gene transcription, and protein synthesis in K562 cells.** A, kinetics of HSF activation by 2-cyclopenten-1-one. Whole-cell extracts prepared at different times after treatment with 500 μM 2-cyclopenten-1-one or 3 h after heat shock (45°C for 20 min) were subjected to EMSA (right panel). Positions of HSF-DNA binding complex (HSF), constitutive HSE binding activity (CHBA), and non-specific protein-DNA interactions (NS) are indicated, as in Fig. 1. The levels of HSF DNA-binding activity in 2-cyclopenten-1-one-treated cells were quantitated by Molecular Dynamics PhosphorImager analysis (left panel). HSF values were normalized to the level of HSF DNA binding activity at 9 h after treatment, which was given a value of 100%. B, transcription rates, measured by nuclear run-on assay. 32P-Labeled mRNA was efficiently translated in K562 cells. Synthesis of HSP70 was sustained for at least 9 h after treatment. Increased expression of this protein was also detected by immunoprecipitation (Fig. 2C, lower panel).

To investigate whether the α,β-unsaturated carbonyl group present in 2-cyclopenten-1-one was the molecular structure responsible for HSF activation, K562 cells were treated with different concentrations of 2-cyclopenten-1-one, cyclopentanone (a similar molecule with a saturated carbonyl), or with cyclopentene, which contains a double bond, but not a carbonyl group. Neither cyclopentanone nor cyclopentene were able to induce HSF DNA binding activity (Fig. 3A), indicating that the presence of an α,β-unsaturated carbonyl is essential for HSF activation. Since the cyclopentenone prostaglandin A1 (PGA1) functions as signal for HSF activation in many types of mammalian cells (7, 10), we compared the effect of PGA1 with either 2-cyclopenten-1-one, or with 1-octen-3-ol and oenanthic acid, whose structure resembles the two aliphatic side chains of PGA1. Neither 1-octen-3-ol nor oenanthic acid were able to induce HSF activation, confirming that the key structural component for HSF activation by prostaglandins is the cyclopentenone moiety (Fig. 3B). However, PGA1 is able to activate HSF at concentrations much lower (approximately 25 times) than 2-cyclopenten-1-one (Fig. 3C). The presence of the aliphatic side chains could be functional by facilitating either entry into cells or detection of the molecular target.

Starting from the early observation that prostaglandins of the A type inhibit Sendai virus replication and prevent the establishment of persistent infections in cultured cells (19), it is now well established that cyclopentenone prostaglandins possess a potent antiviral activity against several DNA and RNA viruses, including the human immunodeficiency virus type 1 (HIV-1) (20), *in vitro* and *in vivo* (reviewed in Refs. 10 and 21). The antiviral activity of a long acting synthetic analogue of PGA1 (di-M-PGA2) has also been shown in *vivo*, in a mouse model infected with influenza A virus (22). In negative-strand RNA virus models prostaglandins of the A and J type provoke a selective and dramatic block of virus protein synthesis (11, 12). This block is exerted at the translational level and is dependent on HSP70 expression in infected cells (reviewed in Ref. 10). A possible model has been hypothesized, in which HSP and virus messages, both of which can be translated in conditions where cellular protein synthesis is impaired, could possess similar mechanisms for preferential translation and could then compete with each other (23).

To investigate whether 2-cyclopenten-1-one could mimic the PG-induced block of virus protein synthesis, epithelial monkey kidney MA104 cells were infected with vesicular stomatitis virus and then treated with 2-cyclopenten-1-one, at concentrations which induced HSP70 synthesis. VSV titers were determined 24 h post-infection by cytopathic effect 50% assay. As shown in Fig. 4A, 2-cyclopenten-1-one was found to inhibit the production of VSV infectious particles in a dose-dependent manner and with a selective index of 36.

As compared with PGA1, which dramatically inhibits VSV replication in MA104 cells at concentrations lower than 50 μM (12, 24), higher concentrations of 2-cyclopenten-1-one were needed to inhibit virus replication. The relative potency of PGA1 and 2-cyclopenten-1-one in activating HSF (Fig. 3) correlates with the antiviral activity, reinforcing the hypothesis that the heat shock response is involved in the control of virus replication (10, 23).

To determine the effect of 2-cyclopenten-1-one on HSP70 induction and virus protein synthesis in MA104 cells, uninfected or VSV-infected cells were treated with 500 μM 2-cyclopenten-1-one, or with control diluent soon after VSV infection,
and labeled with [35S]methionine (8 μCi/2 × 10⁶ cells, 1-h pulse starting 5 h post-infection). Equal amounts of protein were analyzed on 10% SDS-PAGE gels and processed for autoradiography. As shown previously for PGA and PGJ compounds (10–12), 2-cyclopenten-1-one treatment causes a selective and dramatic inhibition of viral protein synthesis, associated with HSP70 induction (Fig. 4

The activation of heat shock genes and a cytoprotective role of HSP70 has been described in a wide variety of human diseases, including ischemia, metabolic disorders, inflammation, and infection (23, 25–31). HSP70 has been shown recently to be critically involved in myocardial protection by ischemia-induced injury also in animals (32). These observations justify the attempt to characterize new HSP inducers, which could be used therapeutically. The results described identify 2-cyclopenten-1-one as a new inducer of HSP70 with antiviral activity and indicate that the presence of an α,β-unsaturated carbonyl group in the cyclopentane ring is essential for induction. As shown previously for other HSP inducers, including antiviral prostaglandins, sodium arsenite, cadmium, and hyperthermia itself (10–12), 2-cyclopenten-1-one-induced HSP70 synthesis is associated with a selective inhibition of virus protein synthesis, suggesting a cytoprotective role of this protein during viral infection. These results open new perspectives in the search for novel HSP inducers, which could be utilized as cytoprotective and antiviral drugs.

In the present report we have also identified the molecular structure of natural prostaglandins responsible for HSF1 acti-
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Cyclopentenone prostaglandins are physiologically present in body fluids and, apart from the antiviral activity, are known to affect cell proliferation and differentiation (reviewed in Refs. 8 and 21). Recently the PGJ metabolite 15-deoxy Δ12,14-PGJ2, which is characterized by the presence of a cyclopentenone ring and activates HSF (33), has been shown to be the natural ligand for the adipocyte determination factor PPARγ, inducing adipocyte differentiation (34, 35). The fact that cyclopentenone prostaglandins activate HSF suggests the possibility that the heat shock response could play a role in physiological processes controlled by prostaglandins.

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