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Anti-Inflammatory Activity of 15-Deoxy-$\Delta^{12,14}$-PGJ_2 and 2-Cyclopenten-1-one: Role of the Heat Shock Response

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ABSTRACT

The transcription factor heat shock factor 1 (HSF1) plays a key role in the expression of several genes, such as heat shock protein (*hsp*) genes, which are cytoprotective against several pathological conditions, including inflammation. Cyclopentenone prostaglandins (cyPG) are able to activate HSF1 and induce the synthesis of the 70-kDa *hsp* (*hsp70*) in mammalian cells. These molecules are characterized by the presence of a reactive α,β-unsatured carbonyl group in the cyclopentane ring (cyclopentenone) which is the key structure for triggering HSF1 activation. In the present study, we investigated, in carrageenin hind-paw edema, an acute model of inflammation, the effect of double-stranded oligodeoxynucleotides with consensus HSF1 sequence as transcription factor decoys to inhibit HSF1 binding to native DNA sites. We show that HSF1 activation and *hsp72* expression occurs in inflamed tissue and that this effect is associated with the remission of the inflammatory reaction. Moreover, we studied the effect of prostaglandin 15-deoxy-$\Delta^{12,14}$-prostaglandin (PG) J_2, of its precursor, PGD_2 and, for the first time *in vivo,* the effect of the cyclopentenone ring structure itself, 2-cyclopenten-1-one. Our results demonstrated that all agents used had anti-inflammatory properties and that this effect was associated with HSF1-induced *hsp72* expression in vivo, suggesting that the use of cyclopentenone derivatives may represent a novel therapeutic approach to the treatment of inflammatory diseases.

Most organisms respond to hyperthermia and other environmental stresses by increasing the synthesis of a group of cytoprotective proteins known as stress proteins or heat shock proteins (*hsp*) (Lindquist and Craig, 1988). Different classes of *hsp* have been described previously according to their molecular masses (i.e., *hsp90, hsp70, hsp60*). The *hsp70* family, one of the most extensively studied in mammalian cells, includes several proteins that function as molecular chaperones (*hsp73*, *hsp72*, etc.) regulating the folding of nascent proteins, the refolding of denatured proteins, and preventing the formation of insoluble cytotoxic protein aggregates (Lindquist and Craig, 1988; Schlesinger, 1990; Santoro, 2000; Pockley, 2002). In eukaryotic cells, *hsp* expression is regulated at transcriptional level via activation of the heat shock transcription factor (HSF), which binds to DNA at a specific site (heat shock element) in the *hsp* gene promoter region (Lis and Wu, 1993; Santoro, 2000). The cloning of HSF genes in higher eukaryotes has revealed a family of HSFs (HSF1-HSF4). HSF type 1 (HSF1) is considered the main stress-responsive activator in mammalian cells. HSF1 is constitutively present in the cytoplasm as an inert monomer bound to *hsp70* and other chaperones. Its activation is induced by various types of stress, including heat shock, anoxia, exposure to toxins and heavy metals that induce HSF oligomerization to a trimeric DNA binding form, and nuclear translocation (Santoro, 2000). The cytoprotective role of *hsp70* has been shown both in vitro and in vivo in a variety of human diseases, including metabolic disorders (Williams et al., 1993), infection (Amici et al., 1994), ischemia (Mestril et al., 1994) and inflammation (Polla et al., 1998; Ianaro et al., 2001a,b; Dybdahl et al., 2002; Van Molle et al., 2002).

It is well known that the inflammatory response involves the sequential activation of various signaling molecules, among which arachidonic acid metabolites, particularly prostaglandins (PG), play an important role. Different types of PG are produced in large amounts by the inducible isoform of cyclooxygenase (COX-2), which is expressed in inflammatory cells and tissues in response to a variety of inflammatory stimuli, cytokines, and mitogens (Seibert et al., 1994). Sev-

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ABBREVIATIONS: hsp, heat shock proteins; HSF, heat shock factor; PG, prostaglandin; COX-2, cyclooxygenase 2; $15dPGJ_2$, 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2; cyPG, cyclopentenone prostaglandins; TFD, transcription factor decoy; wt, wild type; mut, mutant; EMSA, electrophoretic mobility shift assay; NF-κB, nuclear factor κB; cyclopentenone, 2-cyclopenten-1-one.

eral lines of evidence, however, suggest that the function of COX-2 may change during the inflammatory response, contributing to the resolution of inflammation. It has in fact been shown that COX-2 protein expression increases substantially during the resolution of inflammatory reactions, such as murine granulomatous air pouch and rat carrageenin pleurisy (Appleton et al., 1995; Gilroy et al., 1999). Moreover, the increase of COX-2 protein expression was associated with high levels of PGD_2 and the cyclopentenone PGD_2 metabolite of the J-series, such as 15-deoxy-$\Delta^{12,14}$-PGJ2 ($15dPGJ_2$), suggesting a physiological anti-inflammatory role for these molecules in the resolution of inflammatory reactions (Gilroy et al., 1999).

It has been shown that cyclopentenone prostaglandins (cyPG) of the A and J type are able to activate HSF1 and induce the synthesis of *hsp70* in a variety of mammalian cells (Amici et al., 1994; Santoro, 2000). CyPG are characterized by the presence of a reactive α,β-unsatured carbonyl group in the cyclopentane ring (cyclopentenone) that seems to be the key structure for triggering HSF activation. It has in fact been demonstrated that the cyclopentenone ring structure itself, 2-cyclopenten-1-one, specifically induces the expression of *hsp70* through activation of HSF1 in human erythroleukemia cells (Rossi et al., 1996).

In the present study, using rat paw carrageenin edema as a model of acute inflammation, we show that HSF1 activation and *hsp72* expression both actually occur in inflamed tissue and that the remission of the inflammatory reaction is associated with HSF1-dependent *hsp72* expression. Furthermore, we report that administration to animals of $15dPGJ_2$, PGD_2, or 2-cyclopenten-1-one inhibits the inflammatory reaction through the activation of HSF1 and the selective expression of *hsp72*, suggesting a novel therapeutic approach to the treatment of inflammatory diseases.

Materials and Methods

Animals. Male Wistar rats (Harlan, Milan, Italy) weighing 140 to 160 g were used in all experiments. Animals were provided with food and water ad libitum. The light cycle was automatically controlled (on at 7:00 AM; off at 5:00 PM) and the room temperature thermostatically regulated to 22 ± 1°C. Before the experiments, animals were housed in these conditions for 3 to 4 days to become acclimatized. Animal care was in accordance with Italian and European regulations on protection of animals used for experimental and other scientific purposes.

Transcription Factor Decoy Oligonucleotides. The transcription factor decoys (TFDs) used in this study were synthesized as phosphorothioate derivatives, which are resistant to degradation by endonucleases, according to our specifications by Tib Molbiol Roche Biochemical (Monza, Italy). Double-stranded phosphorothioate TFD to HSF1 was prepared by annealing of sense and antisense oligonucleotides in vitro in 1× annealing buffer. The mixture was heated at 100°C for 2 min and allowed to cool to room temperature slowly over 18 h.

The phosphorothioate TFD sequences used in this study were: 5′-CTA GAA GCT TCT AGA AGC TTC TAG-3′ for wild-type HSF (wt-TFD) and 5′-ATA TCC GCA GGT ATC GGC AGT TAG-3′ for mutant HSF TFD (mut-TFD).

Paw Edema. Paw edema was induced by subplantar injection of 0.1 ml of sterile saline containing 1% λ-carrageenin (control group) into the rat right hind paw. The test agents used in this study were: wild type-TFD (3 nmol/paw), mutant TFD (3 nmol/paw), $15dPGJ_2$ (1–10 nmol/paw; Cayman Chemicals, Ann Arbor, MI), PGD_2 (10–100 nmol/paw; Cayman Chemicals), cyclopentenone and cyclopentanone (10–100 nmol/paw; Fluka, Milan, Italy). All these agents were given either concomitantly or at different time points before carrageenin injection (see *Results*). Paw volume was measured by a plethysmometer (Basile, Italy) immediately after the injection as described previously (D'Acquisto et al., 2000). Subsequent readings of the same paw were carried out at 1-h intervals up to 24 h and compared with the initial readings. The increase in paw volume was considered as edema volume. In some experiments, the rats were killed in an atmosphere of CO_2 immediately after the paw volume was measured and soft tissue from each inflamed paw was recovered by scalpel and immediately processed to obtain whole extracts (see below).

Preparation of Whole Tissue Extracts. All the extraction procedures were performed on ice with ice-cold reagents. Whole tissue extracts (resident cells and migrated neutrophils) were prepared by homogenizing the inflamed tissues in 1 ml of a high-salt extraction buffer [20 mM HEPES, pH 7.9, 420 mM NaCl, 1.5 mM MgCl2, 0.2 mM EDTA, 25% (v/v) glycerol, 0.5 mM phenylmethylsulfonyl fluoride, 1.5 μg/ml soybean trypsin inhibitor, 7 μg/ml pepstatin A, 5 μg/ml leupeptin, 0.1 mM benzamidine, and 0.5 mM dithiothreitol; Calbiochem, La Jolla, CA] and incubated on ice for 15 min. After centrifugation at 13,000*g* at 4°C for 5 min, the protein concentration in the supernatant was determined by the Bio-Rad (Milan, Italy) protein assay kit, and then it was aliquoted and stored at −80°C.

Electrophoretic Mobility Shift Assay. Double-stranded oligonucleotides containing the idealized heat shock element (5′-CTA GAA GCT TCT AGA AGC TTC TAG-3′) sequence (Sarge et al., 1991; Tacchini et al., 1997; Ethridge et al., 1998) were end-labeled with [γ-^{32}P]ATP (ICN Biomedicals, Milan, Italy). Aliquots of nuclear extracts (12 μg of protein for each sample) were incubated for 30 min with radiolabeled oligonucleotides (2.5–5.0 × 10^4 cpm) in 20 μl of reaction buffer containing 2 μg of poly dI-dC (Roche, Milan, Italy), 10 mM Tris-HCl, pH 7.5, 100 mM NaCl, 1 mM EDTA, 1 mM DL-dithiothreitol, 1 mg/ml bovine serum albumin (Sigma, Milan, Italy), and 10% (v/v) glycerol. The specificity of the DNA/protein binding was determined for HSF1 by competition reaction in which a 50-fold molar excess of unlabeled wild-type or mutant HSF oligonucleotide, or Oct-1 oligonucleotide was added to the binding reaction 10 min before addition of radiolabeled probe. In supershift assay, antibodies reactive to HSF1 or HSF2 proteins (a kind gift of Dr. R. I. Morimoto) were added to the reaction mixture 30 min before the addition of radiolabeled HSF1 probe. Protein-nucleic acid complexes were resolved by electrophoresis on 4% nondenaturing polyacrylamide gel in 0.5 × Tris-borate/EDTA buffer at 150 V for 2 h at 4°C. The gel was dried and autoradiographed with intensifying screen at −80°C for 20 h. Quantitative evaluation of HSF-heat shock element complex formation was determined by PhosphorImager (Amersham Biosciences, Milan, Italy) analysis.

Western Blot Analysis. The levels of *hsp72* were quantified in whole extract by Western blot analysis as described previously (Ianaro et al., 2001a). Briefly, equivalent amounts of protein (20 μg) from each sample were mixed with gel loading buffer (50 mM Tris/10% SDS/10% glycerol/10% 2-mercaptoethanol/2 mg bromphenol blue per milliliter) in a ratio of 1:1, boiled for 3 min, centrifuged at 10,000*g* for 10 min, and electrophoresed in a 8% discontinuous polyacrylamide minigel. The proteins were transferred onto nitrocellulose membranes according to the manufacturer's instructions. The membranes were saturated by incubation at 4°C overnight with 10% nonfat dry milk (Bio-Rad) in phosphate-buffered saline (ICN Biomedicals) and then incubated with anti-*hsp72* (SPA-812; StressGen, Victoria, BC, Canada), which specifically recognizes the inducible but not the constitutive member of the *hsp70* family polyclonal antibodies, or anti-β-actin (1:1000) mouse antibody for 2 h at room temperature. The membranes were washed three times with 1% Triton X-100 in phosphate-buffered saline and then incubated with anti-mouse immunoglobulins coupled to peroxidase (1:2000; Amersham Biosciences). The immune complexes were visualized by the enhanced chemiluminescence method. Subsequently, the relative

expression of the proteins was quantified by densitometric scanning of the X-ray films with GS-700 imaging densitometer (Bio-Rad) and the Molecular Analyst computer program (IBM, White Plains, NY). β-Actin (Sigma) Western blot analysis was performed to ensure equal sample loading. Recombinant human *hsp72* protein (StressGen) was used as positive control (data not shown).

Statistics. Data are expressed as mean ± S.E.M. for n rats. Statistical analysis was done using analysis of variance followed by a Bonferroni multiple comparison test. The level of statistically significant difference was defined as $p < 0.05$.

Results

Time Course of HSF1 Activation and hsp72 Expression in Rat Carrageenin Paw Edema. Carrageenin injection caused a time-dependent increase of paw volume peaking at 4 h and returning at baseline 24 h after carrageenin challenge (Fig. 1a). To detect HSF/DNA binding activity, whole extracts from tissue of individual rat hind paw injected with carrageenin or saline were collected at different time points after carrageenin challenge (1, 4, 12, and 24 h) and analyzed by EMSA. As shown in Fig. 1b, HSF/DNA binding activity was virtually undetectable in proteins from tissue of saline-treated rats, whereas a retarded band of DNA-protein complex was faintly detected in the early phase (1 h) of paw edema, reached its peak at 4 h, and was still clearly detectable at 12 and 24 h after carrageenin injection (Fig. 1b). The enhanced activation of HSF observed was associated with increased expression of *hsp72* compared with that observed in extracts from tissue collected from saline-treated rats. In fact, Western blot analysis showed a time-dependent increase of *hsp72* protein levels in whole extracts from carrageenin-treated rats beginning at 4 h and reaching higher levels of expression at 12 and 24 h after carrageenin challenge (Fig. 1c).

To confirm the specificity of the HSF/DNA complex activated by carrageenin we performed competition and supershift assays (Fig. 1d). The specificity of the HSF/DNA binding complex was demonstrated by the complete displacement of the HSF/DNA binding in the presence of a 50-fold molar excess of unlabeled HSF probe in the competition reaction. In contrast, a 50-fold molar excess of unlabeled mutated HSF probe or Oct-1 oligonucleotide had no effect on the DNA-binding activity. Furthermore, to establish which member of the HSF family was activated by carrageenin, whole extracts were preincubated with antibodies specific for either HSF1 or HSF2 and analyzed by EMSA. Addition of anti-HSF1 (1:50) but not anti-HSF2 (1:50) to the binding reaction resulted in mobility retardation of the HSF band, thus indicating that carrageenin activates HSF type 1 (Fig. 1d).

Effect of Transcription Factor Decoy on Carrageenin-Induced Paw Edema and on HSF1 Activation and hsp72 Expression in Inflamed Paw Tissue. In saline-treated animals, injection into the rat paw of transcription factor decoys agents at the doses used in this study did not produce any detectable edema (data not shown). In carrageenin-treated animals, coinjection of carrageenin with the

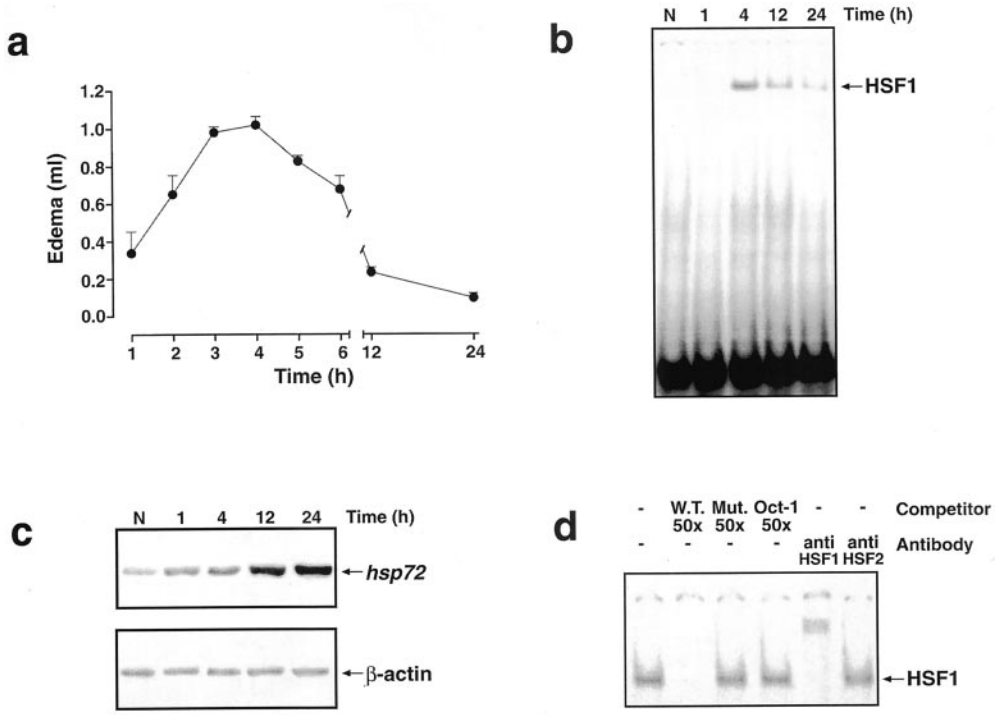

Fig. 1. Time course of rat carrageenin paw edema, HSF1/DNA binding activity, and *hsp72* protein expression. a, rat paw volume was evaluated at different time points after carrageenin injection. The values are expressed as mean ± S.E.M. where n = 7 to 10 rats. b, time course of carrageenin-induced HSF1/DNA binding activity. EMSA was performed on whole extracts of soft tissue from rat hind paw of saline-treated rats (N) or from carrageenin-treated rats at different time points (1, 4, 12, and 24 h) after injection and is representative of one paw of five or six analyzed (top). c, time course of *hsp72* expression. Immunoblot analysis was performed on whole extracts of tissue from rat hind paw of either saline-treated rats (N) or carrageenin-treated rats at different time points (1, 4, 12, and 24 h) after injection. Equal loading was confirmed by β-actin staining. Data are representative of one paw of five or six analyzed. d, characterization of carrageenin-induced HSF1/DNA binding activity. In competition reactions, whole extracts were incubated with radiolabeled HSF probe in the absence or presence of 50-fold molar excess of unlabeled HSF probe (W.T. 50×), mutated nonfunctional HSF probe (Mut. 50×), or an oligonucleotide containing the consensus sequence for Oct-1 (Oct-1 50×). In supershift experiments, nuclear extracts were incubated with antibodies against HSF1 or HSF2 30 min before incubation with the radiolabeled HSF probe.

HSF decoy (wt-TFD, 3 nmol/paw) did not significantly modify edema formation up to 4 h after carrageenin injection, although it significantly exacerbated paw edema at later times (Fig. 2a). In contrast, coinjection of mutant TFD (mut-TFD, 3 nmol/paw; $n = 6$) had no effect (Fig. 2a).

To evaluate the effect of TFD on HSF1/DNA binding activity and *hsp72* protein expression, individual rat hind paws were recovered at different times after subplantar injection, and whole extracts from tissue of each rat hind paw were analyzed by EMSA and Western blot. Treatment of rats with wt-TFD greatly inhibited HSF1/DNA binding activity at all the time points; in contrast, mut-TFD did not modify carrageenin-induced HSF1/DNA binding activity compared with control animals (Fig. 2b). The levels of *hsp72* in tissue extracts from rat hind paws collected at different times after carrageenin injection were examined by immunoblot analysis. As described in the previous paragraph (Fig. 1c), an increase of *hsp72* levels over the constitutive control levels is evident at 12 and 24 h after carrageenin injection. Administration of wt-TFD greatly reduced *hsp72* accumulation, whereas mut-TFD had no effect on the carrageenin-induced increase of *hsp72* levels (Fig. 2c).

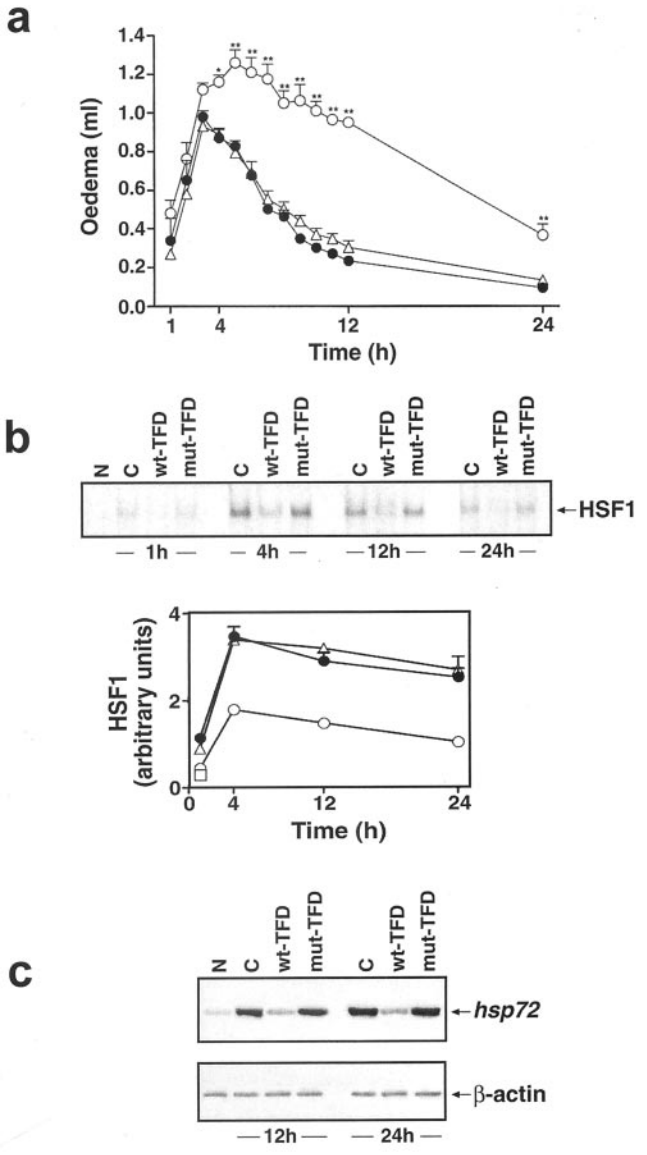

Fig. 2. Effect of TFD on rat carrageenin edema, HSF1/DNA binding activity, and *hsp72* protein expression. a, effect of wild-type TFD (3 nmol/paw; ○) or mutant TFD (3 nmol/paw; △) on paw edema induced by carrageenin. ●, edema induced by carrageenin alone (control group). The results are expressed as mean ± S.E.M., where $n = 5$ to 8 rats. *, $p < 0.05$; **, $p < 0.01$, versus control group. b, effect of TFD on HSF1/DNA-binding activity. EMSA was performed on whole extracts from saline-injected rat hind paw (N) or from carrageenin-injected rat hind paw harvested at different time points (1, 4, 12. and 24 h) after carrageenin injection from animals untreated (C), treated with wild-type TFD (wt-TFD) or with mutant TFD (mut-TFD). EMSA is representative of one paw of five or six analyzed (top). Quantitative evaluation of HSF1/DNA-binding activity was determined by PhosphorImager analysis (bottom). Symbols for different groups are the same as in a. c, effect of TFD on *hsp72* protein expression. Immunoblot analysis was performed on whole extracts from saline-injected rat hind paw (N) or carrageenin-injected rat hind paw from animals untreated (C), treated with wild-type TFD (wt-TFD) or with mutant TFD (mut-TFD) at 12 and 24 h after carrageenin injection. Equal loading was confirmed by β-actin staining. Data are representative of one paw of five or six analyzed.

Effect of $15dPGJ_2$ and PGD_2 on Carrageenin-Induced Paw Edema and on HSF1 Activation and hsp72 Expression in Inflamed Paw Tissue. Injection of the active metabolite of PGD_2, $15dPGJ_2$, to a dose of 10 nmol/paw in the absence of carrageenin did not induce any appreciable edema (data not shown). When rats were treated with $15dPGJ_2$ (10 nmol/paw) 1 h before carrageenin challenge, carrageenin-induced paw edema was significantly reduced (by 44%; $p < 0.01$, $n = 8$). Lower doses of $15dPGJ_2$ (1 and 3 nmol/paw) were still effective inhibiting carrageenin-induced paw edema, which was reduced by 8% ($n = 5$) and 19% ($p < 0.05$, $n = 8$) respectively. Coinjection of $15dPGJ_2$ (10 nmol/paw) with wt-TFD (3 nmol/paw) into the rat paw reversed the inhibitory effect exhibited by the cyclopentenone prostaglandin. In contrast mut-TFD failed to modify $15dPGJ_2$ activity (Fig. 3a). The anti-inflammatory effect of $15dPGJ_2$ was associated with a significant increase of both carrageenin-induced HSF1/DNA binding activity and *hsp72* protein expression (Fig. 3, b and c). This increase was abolished when $15dPGJ_2$ was coinjected with wt-TFD (3 nmol/paw; $n = 6$) but not with mut-TFD (3 nmol/paw; $n = 5$) (Fig. 3, b and c), indicating that HSF1 activation is necessary for the anti-inflammatory activity to occur.

The effect of the $15dPGJ_2$ precursor PGD_2 was also analyzed. In preliminary experiments, the most effective anti-inflammatory activity was obtained when PGD_2 was injected 3 h before carrageenin. As shown in Fig. 4a, PGD_2 injection into the rat hind paw caused a small edema 1 h after the challenge. This was attributed to the well known vasodilating properties of PGD_2. However, the modest increase of the paw volume disappeared in the subsequent 2 h. The carrageenin-induced edema was significantly inhibited (by 57%; $p < 0.01$, $n = 8$) by 100 nmol/paw PGD_2 at the peak of the inflammatory reaction (4 h). When PGD_2 (100 nmol/paw) was coinjected with wt-TFD (3 nmol/paw), a complete reversion of the anti-inflammatory effect of the prostaglandin was observed (Fig. 4a). In contrast, mut-TFD (3 nmol/paw; $n = 6$) did not alter the effect of PGD_2 (Fig. 4a).

Treatment of rats with PGD_2 (100 nmol/paw; $n = 9$) caused a significant increase of both carrageenin-induced HSF1/DNA binding activity and *hsp72* protein expression (Fig. 4, b and c). As in the case of $15dPGJ_2$, these increases were completely abolished when PGD_2 was coinjected with wt-TFD (3 nmol/paw; $n = 6$), but not with mut-TFD (3 nmol/paw; $n = 5$) (Fig. 4, b and c).

Cyclopentenone Inhibits Carrageenin-Induced Paw Edema Formation and Induces HSF1 Activation and hsp72 Expression in Inflamed Paw Tissue. It has been previously shown that 2-cyclopenten-1-one (cyclopentenone) is able to induce HSF1 activation and *hsp72* expression in human cells in vitro, even though at concentrations much higher than the natural cyclopentenone prostaglandins (Rossi et al., 1996). We have then investigated whether cy-

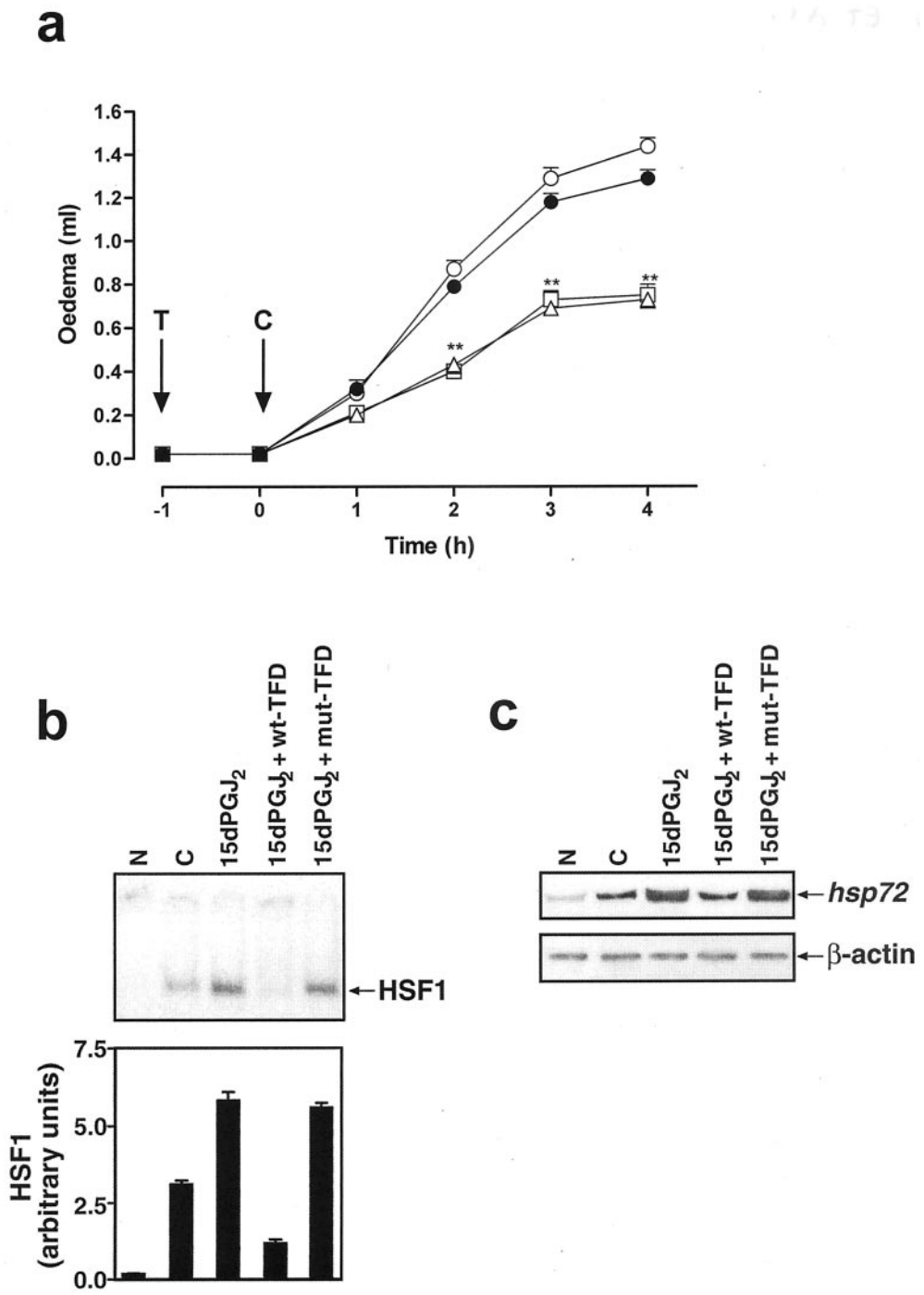

Fig. 3. Inhibition of rat carrageenin edema by $15dPGJ_2$ is associated with enhancement of HSF1/DNA binding activity and *hsp72* protein expression. a, $15dPGJ_2$ alone (10 nmol/paw; △) or in combination with either wild-type TFD (3 nmol/paw, ○) or mutant TFD (3 nmol/paw; □) was injected into the rat hind paw 1 h before carrageenin injection. ●, edema induced by carrageenin alone (control group). The results are expressed as mean ± S.E.M., where $n = 5$ to 8 rats. **, $p < 0.01$, versus control group. T, time of test agent injection; C, time of carrageenin injection. b, the effect of $15dPGJ_2$ (10 nmol/paw) alone or coinjected either with wild-type (wt) or mutant (mut) (3 nmol/paw) TFD on HSF1/DNA binding activity was evaluated by EMSA in soft tissue from rat hind paw 4 h after carrageenin injection (top). Data shown are representative of one paw of five or six analyzed. N, saline injected animals; C, control group. Quantitative evaluation of HSF1/DNA-binding activity was determined by PhosphorImager analysis (bottom). c, the effect of $15dPGJ_2$ (10 nmol/paw) alone or coinjected with either wild-type (wt) or mutant (mut) TFD (3 nmol/paw) on *hsp72* protein expression was evaluated by Western blot analysis in the extracts described in b. Equal loading was confirmed by β-actin staining. Data are representative of one paw of five or six analyzed.

clopentenone is able to mimic the effect of $15dPGJ_2$ in our in vivo model. In saline-treated animals, the injection into the rat paw of cyclopentenone at the doses used in this study did not produce any detectable edema. In carrageenin-treated animals, treatment with cyclopentenone (10, 30, and 100 nmol/paw) 3 h before carrageenin challenge significantly inhibited edema formation in a dose-related fashion (Fig. 5). Maximal anti-inflammatory effect was obtained at the dose of 100 nmol/paw (60%; $p < 0.001$, $n = 10$). (Fig. 6b). As shown above for the natural cyclopentenone prostaglandins, when cyclopentenone (100 nmol/paw) was coinjected with wt-TFD (3 nmol/paw), a complete reversion of its anti-inflammatory effect was observed, whereas mut-TFD (3nmol/paw; $n = 6$) had no effect (Fig. 6b).

Treatment of rats with cyclopentenone (100 nmol/paw; $n = 9$) caused a significant increase of both carrageenin-induced HSF1/DNA binding activity and *hsp72* protein expression (Fig. 6, b and c). These increases were completely abolished when cyclopentenone was coinjected with wt-TFD (3 nmol/paw; $n = 9$) but not with mut-TFD (3 nmol/paw; $n = 6$) (Fig. 6, b and c), indicating that HSF1 activation is essential for the cyclopentenone-induced anti-inflammatory effect. To fur-

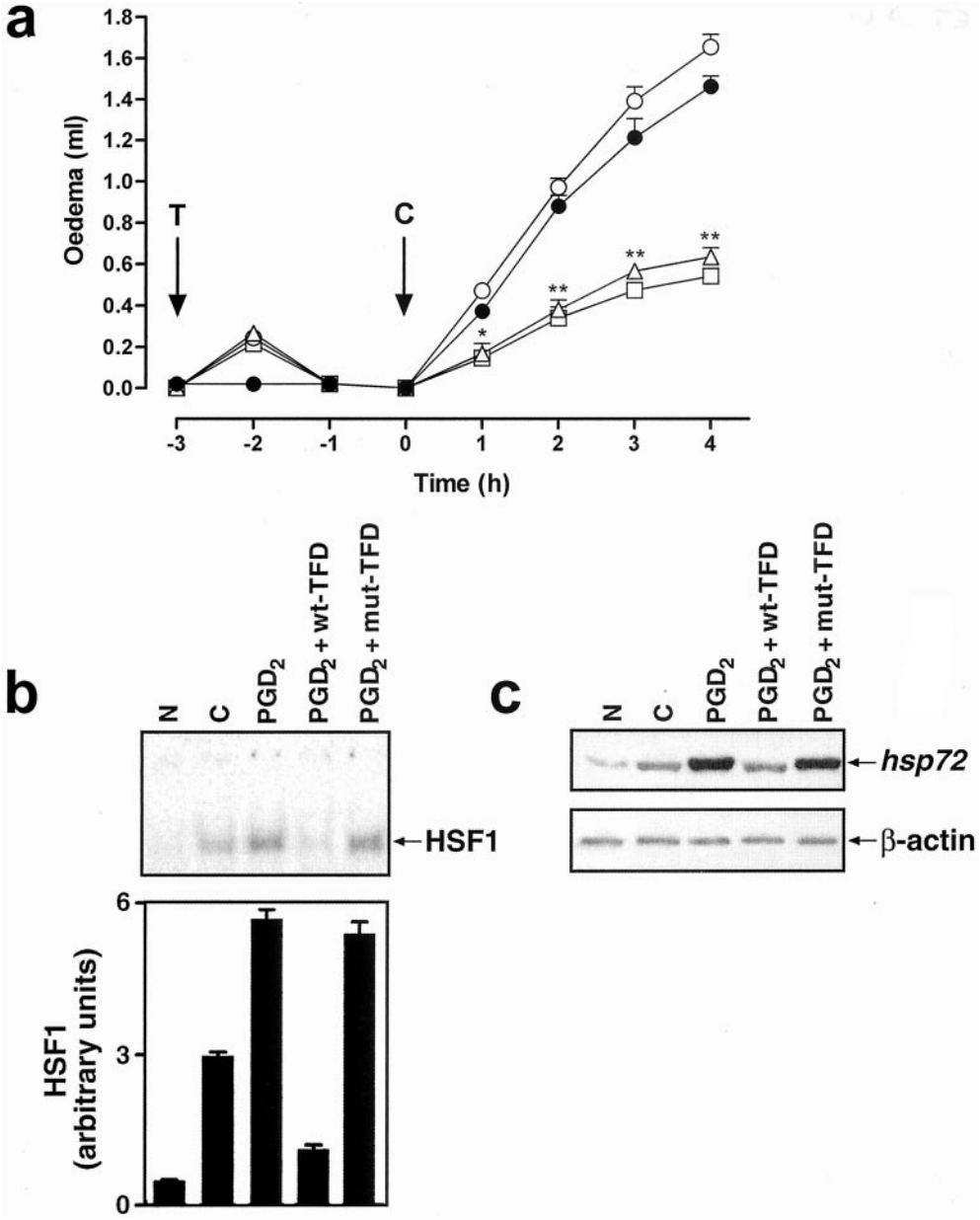

Fig. 4. Effect of PGD_2 on rat carrageenin edema, HSF1/DNA binding activity and *hsp72* protein expression. a, PGD_2 alone (100 nmol/paw; △) or in combination with either wild-type TFD (3 nmol/paw; ○) or mutant TFD (3 nmol/paw; □) was injected into the rat hind paw 3 h before carrageenin injection. ●, edema induced by carrageenin alone (control group). The results are expressed as mean ± S.E.M., where $n = 5$ to 8 rats. *, $p < 0.05$; **, $p < 0.01$ versus control group. T, time of test agent injection; C, time of carrageenin injection. b, the effect of PGD_2 (100 nmol/paw) alone or coinjected with either wild-type (wt) or mutant (mut) TFD (3 nmol/paw) on HSF1/DNA binding activity was evaluated by EMSA in soft tissue from rat hind paw 4 h after carrageenin injection (top). Data shown are representative of one paw of five or six analyzed. N, saline injected animals; C, control group. Quantitative evaluation of HSF1/DNA-binding activity was determined by PhosphorImager analysis (bottom). c, the effect of PGD_2 alone or coinjected with either wild-type (wt) or mutant (mut) TFD on *hsp72* protein expression was evaluated by Western blot analysis in the extracts described in b. Equal loading was confirmed by β-actin staining. Data shown are representative of one paw of five or six analyzed.

ther verify this hypothesis, we tested the effect of cyclopentanone, which was previously shown to be unable to activate HSF1 in human cells (Rossi et al., 1996). As shown in Fig. 6, cyclopentanone administered at the same dose and at the same time as cyclopentenone had no effect on either carrageenin-induced edema (Fig. 6b) or carrageenin-induced HSF1/DNA binding activity and *hsp72* protein expression (Fig. 6, c and d).

Discussion

Heat shock proteins are used by eukaryotic cells in the repair process after different types of injury, preventing damage resulting from the accumulation and aggregation of non-native proteins (Morimoto and Santoro, 1998). In vertebrates *hsp*, and *hsp70* in particular, is considered a part of a protective mechanism against certain pathological conditions, including ischemic damage, infection, and inflammation (Pockley, 2002). In the case of inflammation, a protective role of *hsp* has been shown in a variety of experimental model (Jattela et al., 1992; Morris et al., 1995; Ianaro et al., 2001a; Van Molle et al., 2002). In the present study, we demonstrate, using a transcription factor decoy strategy, that carrageenin injection causes HSF1-induced *hsp72* expression in the inflamed tissues and that activation of the heat shock response seems to be closely associated with the remission of the inflammatory reaction. The results of this study confirm and strengthen our previous findings showing that *hsp* genes may function as anti-inflammatory or "therapeutic" genes, and their selective in vivo transactivation may lead to remission of the inflammatory reaction (Ianaro et al., 2001a,b).

It has been shown that cyclopentenone prostaglandins are produced at the late stage of an inflammatory reaction, and it has been suggested that they may contribute to the resolution of inflammation (Gilroy et al., 1999; Ianaro et al., 2001b). An anti-inflammatory activity of cyclopentenone prostaglandins, and of $15dPGJ_2$ in particular, has been reported in several models of short- and long-term inflammation (Su et al., 1999; Kawahito et al., 2000; Ianaro et al., 2001b). The results shown in the present report demonstrate a strong anti-inflammatory activity of $15dPGJ_2$ and its precursor PGD_2, also in the rat paw carrageenin edema model of short-term inflammation. $15dPGJ_2$ and PGD_2 showed a different kinetic of HSF1 activation that was correlated with their anti-inflammatory activity. $15dPGJ_2$ was effective when it was administered 1 h before carrageenin, whereas the precursor PGD_2 behaves differently; if coinjected or injected 1 h before carrageenin, PGD_2 induced, in the first hour after injection, a greater edema than carrageenin alone, probably because of the well known vasodilating properties of this prostaglandin. This effect was substituted, in the subsequent hours, by an inhibition of carrageenin-induced paw edema

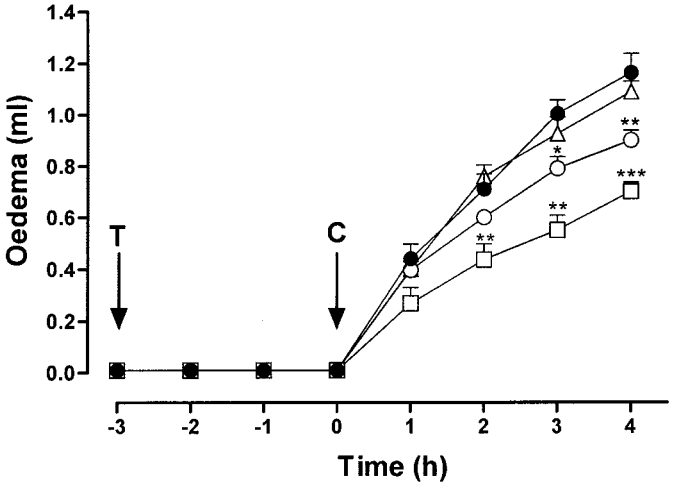

Fig. 5. Dose-dependent inhibition of rat carrageenin-induced edema by 2-cyclopenten-1-one. Effect of 2-cyclopenten-1-one at 10 (△), 30 (○), and 100 nmol/paw (□) on paw edema induced by carrageenin. ●, edema induced by carrageenin alone (control group). The results are expressed as mean ± S.E.M. where $n = 7$ to 10 rats. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ versus control group. T, time of test agent injection; C, time of carrageenin injection.

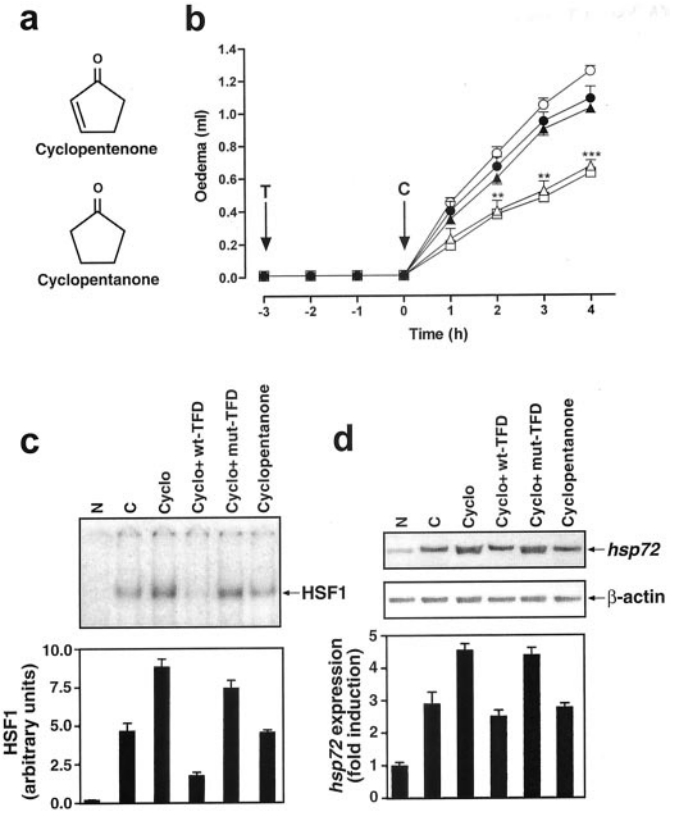

Fig. 6. Effect of cyclopentenone and cyclopentanone on rat carrageenin edema, HSF1/DNA binding activity and *hsp72* protein expression. a, structures of cyclopentenone and cyclopentanone. b, cyclopentenone alone (100 nmol/paw; △) or in combination with either wild-type TFD (3 nmol/paw; ○) or mutant-TFD (3 nmol/paw; □), and cyclopentanone (100 nmol/paw; ▲) were injected into the rat hind paw 3 h before carrageenin injection. ●, edema induced by carrageenin alone (control group). The results are expressed as mean ± S.E.M., where $n = 6$ to 9 rats. **, $p < 0.01$; ***, $p < 0.001$ versus control group. T, time of test agent injection; C, time of carrageenin injection. c, the effect of cyclopentenone (Cyclo, 100 nmol/paw), alone or coinjected with either wild-type (wt) or mutant (mut) TFD (3 nmol/paw), and of cyclopentanone (100 nmol/paw) on HSF1/DNA binding activity was evaluated by EMSA in soft tissue from rat hind paw 4 h after carrageenin injection (upper panel). Data are representative of one paw out of six to eight analyzed. N, saline injected animals; C, control group. Quantitative evaluation of HSF1/DNA-binding activity was determined by PhosphorImager analysis (bottom). d, the effect of cyclopentenone alone or coinjected with either wild-type (wt) or mutant (mut) TFD and of cyclopentanone on *hsp72* protein expression was evaluated by Western blot analysis in the extracts described in c (top). Equal loading was confirmed by β-actin staining (middle). Data are representative of one paw out of five to six analyzed. Quantitative evaluation of *hsp72* protein expression was determined by laser densitometry (bottom). *Hsp72* levels in arbitrary units are expressed as fold-induction of *hsp72* levels in saline-injected rat paws.

(data not shown). When injected 3 h before carrageenin challenge, PGD_2 caused a transient and modest increase of the paw volume 1 h after the challenge. This effect disappeared in the subsequent 2 h and was followed by a pronounced anti-inflammatory activity. These results are in accordance with the hypothesis that the anti-inflammatory effect exhibited by PGD_2 is dependent on its rapid conversion by cells into the active metabolite 15dPGJ2 (Haberl et al., 1998).

The anti-inflammatory effect of the prostaglandins was associated with activation of HSF1 and accumulation of elevated levels of *hsp72* in the inflamed tissue. In addition, prostaglandin-induced inhibition of edema formation seems to depend on activation of the HSF1/*hsp72* pathway, as indicated by the fact that the therapeutic effect was hindered by coinjection of these compounds with the wild type, whereas coinjection with a mutated-TFD had no effect.

It has been previously shown that the cyclopentenone ring is the molecular structure responsible for the activation of HSF1 by cyclopentenone prostaglandins, and that 2-cyclopenten-1-one alone is able to induce HSF1 activation and selective expression of the *hsp70* gene in human cells in vitro, even though at concentrations much higher (approximately 10-fold higher) than the natural prostaglandins (Rossi et al., 1996). We have now shown, for the first time *in vivo*, that injection into the rat paw of 2-cyclopenten-1-one at the dose of 100 nmol/paw has a potent anti-inflammatory effect, inhibiting edema formation by 50 to 60% in carrageenin-treated rats. The anti-inflammatory effect was associated with activation of HSF1 and accumulation of elevated levels of *hsp72* in the inflamed tissue. In contrast, cyclopentanone, which lacks the α,β-unsatured carbonyl group in the cyclopentane ring and is unable to activate HSF1 in human cells (Rossi et al., 1996), had no effect on either carrageenin-induced HSF1/DNA binding activity and *hsp72* protein expression or carrageenin-induced edema. The fact that the effect of 2-cyclopenten-1-one was abolished by coinjection with wild type but not with mutated TFD to HSF1 and by consequent block of HSF1 activation indicates that the anti-inflammatory effect of this compound is dependent on the induction of the heat shock response and expression of heat shock proteins.

It has been hypothesized that cyPG exerts anti-inflammatory activity through the activation of peroxisome proliferator-activated receptor-γ (Jiang et al., 1998; Li et al., 1998) and the inhibition of NF-κB, a transcription factor that has a critical role in the control of the inflammatory response by regulating the expression of a variety of chemotactic and inflammatory cytokines, cytokine receptors, and enzymes involved in the synthesis of pro-inflammatory mediators, such as COX-2 and inducible nitric-oxide synthase (Ghosh et al., 1998). Cyclopentenone prostaglandins in fact potently inhibit NF-κB by blocking the phosphorylation and preventing the degradation of the NF-κB inhibitor IκB-α (Rossi et al., 1997). The I-κB kinase has been recently identified as the molecular target for cyPGs that can directly bind to a cysteine residue (Cys-179) in the activation loop of the β subunit of I-κB kinase (Rossi et al., 2000). Interestingly, it has been shown that inhibition of NF-κB is associated with activation of HSF1 and induction of the heat shock response in a variety of experimental models (Rossi et al., 1997, 1998), and the possibility of cross-talk between these two transcription factors has been suggested (Rossi et al., 1997; Morimoto and Santoro, 1998; Santoro, 2000). The results shown in the present report indicate that the anti-inflammatory activity of cyPG is dependent on their ability to activate the heat shock response and induce the synthesis of cytoprotective heat shock proteins in vivo. However, the possibility cannot be excluded that NF-κB inhibition and other mechanisms may participate in establishing an anti-inflammatory state.

Pharmacological control of inflammation, either in experimental studies or in clinical therapies, rely on the use of anti-inflammatory drugs, such as nonsteroidal anti-inflammatory drugs or glucocorticoids, mainly concentrated on switching off pro-inflammatory systems (e.g., NF-κB, COX-2 etc.). However, it is well known that the ability to mount an inflammatory response is essential for self-protection of living organisms from chemical or physical injuries as well as against bacteria or viruses aggression. Nevertheless, little attention has been so far paid to the understanding of the molecular mechanisms leading to resolution of inflammation, which, in our opinion, is a more physiological approach to the problem. The results of this study suggest that the use of molecules, such as cyclopentenone derivatives, able to switch on endogenous anti-inflammatory systems, such as the HSF1/*hsp72* pathway, may represent a novel strategy for designing more physiological and less harmful anti-inflammatory drugs.

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