Role of cyclopentenone prostaglandins in rat carrageenin pleurisy

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Abstract In this study, using rat carrageenin-induced pleurisy, we found that treatment of rats with either indomethacin or NS-398 suppressed the pleurisy at 2 h but significantly exacerbated this reaction at 48 h. Exacerbated inflammation was associated with reduced prostaglandin D_2 levels, decreased heat shock factor 1 (HSF1) activation, reduced *hsp72* expression and increased activation of nuclear factor κB (NF-κB). Replacement of cyclopentenone prostaglandins by treating rats with either prostaglandin J_2 or prostaglandin D_2 reversed the exacerbating effects of cyclooxygenase inhibitors leading to the resolution of the reaction. In conclusion, we demonstrate that cyclopentenone prostaglandins may act as anti-inflammatory mediators by inducing in inflammatory cells HSF1-dependent *hsp72* expression and NF-κB inhibition, two crucial events for the remission of inflammation.

Key words: Carrageenin pleurisy; Cyclooxygenase inhibitor; Heat shock factor; Heat shock protein; Nuclear factor-κB; Transcription factor

1. Introduction

Carrageenin-induced pleurisy in rats is an experimental model of acute inflammation characterized by plasma extravasation and migration of phagocytic cells. Polymorphonuclear leukocytes are the predominant cell type up to 12 h, then they are replaced by migrating mononuclear cells, which differentiate into macrophages and dominate the reaction up to resolution at 48 h [1,2]. These inflammatory cells synthesize and release a rather intricate cascade of mediators of inflammation among which prostaglandins (PGs) play a relevant role. PGs are a family of signaling molecules synthesized from arachidonic acid by the enzyme cyclooxygenase (COX). COX occurs in two isoforms, COX-1 and COX-2. Generally, COX-1 is constitutively expressed in most tissues, where it modulates physiological processes [3], while inducible COX-2 is considered a pro-inflammatory enzyme and a chief target for the treatment of inflammatory disease [4]. However, it has recently been shown that COX-2 may have anti-inflammatory properties. In fact, it has been found that in the early polymorphonuclear leukocytes-dominated phase of rat carrageenin-induced pleurisy COX-2 mainly catalyzes the synthesis of pro-inflammatory prostaglandins (in particular PGE_2) while in the late mononuclear cells-dominated stage of this inflammatory reaction COX-2 directs the synthesis of cyclopentenone prostaglandins (cyPGs), suggesting a physiological anti-inflammatory role for these molecules in the resolution of an inflammatory reaction [5]. It has been hypothesized that cyPGs exert anti-inflammatory activity through the activation of peroxisome proliferator-activated receptor-γ (PPAR-γ), a nuclear receptor that interferes with the transcriptional activity of the transcription factor nuclear factor-κB (NF-κB) [6,7]. However, recent in vitro studies have shown that cyPGs are able to inhibit the phosphorylation and prevent the degradation of the NF-κB inhibitor IκB-α in human cells expressing very low levels of PPAR-γ [8].

Recent in vitro studies have shown that cyPGs in a non-stressful situation selectively induce in mammalian cells the expression of specific heat shock genes [9]. Induction is mediated by the activation and translocation to the nucleus of a transregulatory protein, the heat shock transcription factor (HSF), which binds to multiple arrays of heat shock elements located in the promoter region of heat shock genes [10]. HSF activation requires trimerization, acquisition of DNA binding activity, localization to the nucleus, and inducible serine phosphorylation resulting in stress-induced transcription of heat shock proteins (*hsp*) [11]. Heat shock proteins have been known for several years to protect cells against a wide variety of toxic conditions, including extreme temperatures, oxidative stress and virus infection [12]. Moreover, we have recently shown that HSF1 is activated in vivo in the late stage of rat carrageenin pleurisy and drives the inflammatory reaction to the remission since its selective inhibition by double-stranded oligodeoxynucleotide caused exacerbation of the pleurisy [13]. In the present study, we demonstrate that in rat carrageenin-induced pleurisy cyPGs may act as endogenous anti-inflammatory mediators by activating the HSF1/*hsp72* pathway and inhibiting NF-κB activation in the cells collected from pleural exudate at the late stage of the inflammatory reaction. Furthermore, we show that the exacerbated pleurisy, consequent on the treatment of animals with indomethacin or NS-398, is associated to a lack of activation of the HSF1/*hsp72* pathway as well as to a prolonged NF-κB activation.

2. Materials and methods

2.1. Animals

Male Wistar rats (Harlan) weighing 175–200 g were used in all experiments. Animal care was in accordance with Italian and Euro-

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Abbreviations: COX, cyclooxygenase; cyPGs, cyclopentenone prostaglandins; EMSA, electrophoretic mobility shift assay; HSF1, heat shock factor 1; *hsp72*, heat shock protein 72; NSAIDs, non-steroidal anti-inflammatory drugs; NF-κB, nuclear factor κB; PPAR-γ, peroxisome proliferator-activated receptor-γ; PGs, prostaglandins

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pean regulations on the protection of animals used for experimental and other scientific purposes.

2.2. Induction of pleurisy

Carrageenin pleurisy induction, measurement of pleural exudate and inflammatory cell collection were carried out as previously described [13].

2.3. Treatments

In a first set of experiments, groups of rats were treated with 0.1, 1.0 or 10 mg/kg NS-398 (Cayman Chemical) or 0.3, 1.0 or 3.0 mg/kg indomethacin (Sigma), both dissolved in polyethyleneglycol (Sigma) and administered p.o. by gastric gavage (0.5 ml/rat) 1 h before carrageenin injection. Two hours after the induction of pleurisy pleural exudates were collected and processed as described [13].

In a second set of experiments both NS-398 (1, 3 or 10 mg/kg) and indomethacin (0.3, 1 or 3 mg/kg) were given p.o. 24 h after induction of pleurisy and every 6 h thereafter. NS-398 was used at a wider dosing range at 2 h than at 48 h since it was more anti-inflammatory early. Some groups of animals, treated with NS-398 (10 mg/kg) or indomethacin (3 mg/kg) 24 h after carrageenin injection as previously described, were also injected into the pleural cavity with PGJ_2 or PGD_2 (Cayman Chemical) both given at 100 μg/kg. In this set of experiments animals were killed 48 h after induction of pleurisy and pleural exudates were collected and processed as described [13]. In each set of experiments the control group received only vehicle(s) by the appropriate route of administration.

2.4. Assay for PGE_2 and PGD_2

PGE_2 and PGD_2 amounts in the supernatant of centrifuged exudate were measured by enzyme immunoassay (Cayman Chemical).

2.5. Preparation of cell extracts

All the extraction procedures were performed on ice with ice-cold reagents. Cells were separated from the inflammatory exudate by centrifugation at $800\times g$ for 10 min at 4°C. Inflammatory cell pellets were washed twice in phosphate-buffered saline (ICN Biomedicals) and centrifuged at $800\times g$ for 5 min at 4°C. Whole-cell extracts were prepared by resuspending the pellet in one packed cell volume of a high-salt extraction buffer and incubated on ice for 15 min. After centrifugation at $13\,000\times g$ at 4°C for 5 min, the protein concentration in the supernatant was determined by the Bio-Rad protein assay kit (Bio-Rad) and then it was aliquoted and stored at −80°C. Nuclear extracts were prepared as previously described [14]. Protein concentration was determined by the Bio-Rad protein assay kit (Bio-Rad).

2.6. Electrophoretic mobility shift assay (EMSA)

Double-stranded oligonucleotides containing the NF-κB recognition sequence (5′-GAT CGA GGG GAC TTT CCC TAG-3′) or the idealized heat shock element (5′-CTA GAA GCT TCT AGA AGC TTC TAG-3′) sequence [15–17] were end-labeled with γ-[^{32}P]ATP (ICN Biomedicals). Aliquots of nuclear extracts (15 μg of protein for each sample) were incubated for 30 min with radiolabeled oligonucleotides ($2.5–5.0\times10^4$ cpm) in 20 μl reaction buffer containing 2 μg poly dI-dC, 10 mM Tris–HCl (pH 7.5), 100 mM NaCl, 1 mM ethylenediaminetetraacetic acid, 1 mM DL-dithiothreitol, 1 mg/ml bovine serum albumin, 10% v/v glycerol. The specificity of the DNA/protein binding was determined for NF-κB by competition reaction in which a 50-fold molar excess of unlabeled wild-type, mutant or Sp-1 oligonucleotide was added to the binding reaction 10 min before addition of radiolabeled probe. In supershift assay, antibodies reactive to p50, p65 or c-Rel proteins (Santa Cruz Biotechnology) were added to the reaction mixture 30 min before the addition of radiolabeled NF-κB probe. 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, mutant or Oct-1 oligonucleotide was added to the binding reaction 10 min before addition of radiolabeled probe. In supershift assay, antibodies reactive to HSF1 (StressGen) or HSF2 (LabVision) proteins 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% non-denaturing polyacrylamide gel in 0.5× Tris borate ethylenediaminetetraacetic acid buffer at 150 V for 2 h at 4°C. The gel was dried and autoradiographed with an intensifying screen at −80°C for 20 h. Subsequently, the relative bands were quantified by densitometric scanning of the X-ray films with a GS-700 Imaging Densitometer (Bio-Rad) and a computer program (Molecular Analyst, IBM).

2.7. Immunoprecipitation and Western blot analysis

The levels of p50, p65 and HSF1 were quantified in nuclear extracts, while the level of *hsp72* was quantified in whole-cell extracts, by immunoprecipitation followed by Western blot analysis according to the manufacturer's instruction (Santa Cruz Biotechnology). Briefly, equivalent amounts of whole cell extracts (100 μg for each sample) were mixed with 40 μl of protein A-Sepharose and 2 μl of anti-p65 (sc-109, Santa Cruz Biotechnology), anti-p50 (sc-8414, Santa Cruz Biotechnology), anti-HSF1 (SPA-901, StressGen), or anti-*hsp72* (SPA-812, StressGen) which specifically recognizes the inducible but not the constitutive member of the hsp70 family polyclonal antibodies, and left overnight at 4°C with continuous shaking. Precipitated immunocomplexes were electrophoresed, transferred onto nitrocellulose membranes and then incubated with anti-p65, anti-p50 and anti-*hsp72* antibodies for 1 h at room temperature. Precipitated HSF1 was incubated with an anti-phosphoserine mouse monoclonal antibody (Sigma Chemicals) as previously described [18]. Recombinant human HSF1 protein and recombinant human *hsp72* protein (StressGen) were used as positive controls. The membranes were then incubated with anti-rabbit or anti-mouse immunoglobulins coupled to peroxidase. The immunocomplexes were visualized by the ECL chemiluminescence method (Amersham). Subsequently, the relative expression of the proteins was quantified by densitometric scanning of the X-ray films with a GS-700 Imaging Densitometer (Bio-Rad) and a computer program (Molecular Analyst, IBM). β-Actin (Sigma) Western blot analysis was performed to ensure equal sample loading.

2.8. Statistics

Data are expressed as mean ± S.E.M. for *n* rats. Statistical analysis was done using an analysis of variance followed by a Bonferroni

Table 1
Effect of COX inhibitors on rat carrageenin-induced pleurisy

Treatment (mg/kg/p.o.)	Exudate at 2 h					Exudate at 48 h				
	PGD_2 (pg/ml)	PGE_2 (pg/ml)	Volume (ml)	Cells/rat ($\times10^6$)	*n*	PGD_2 (pg/ml)	PGE_2 (pg/ml)	Volume (ml)	Cells/rat ($\times10^6$)	*n*
None	422 ± 25	3122 ± 224	0.48 ± 0.03	10.4 ± 0.5	10	1444 ± 95	ND	0.25 ± 0.04	33.1 ± 5.8	10
Indomethacin										
0.3	243 ± 39***	2253 ± 166***	0.26 ± 0.02***	7.7 ± 0.5**	6	978 ± 48***	ND	0.26 ± 0.04	33.9 ± 4.3	7
1	190 ± 29***	1340 ± 80***	0.22 ± 0.05***	4.9 ± 0.5***	7	455 ± 22***	ND	0.32 ± 0.05	47.8 ± 3.1	8
3	ND	424 ± 73***	0.12 ± 0.02***	3.0 ± 0.4***	7	156 ± 09***	ND	1.52 ± 0.10***	81.2 ± 4.2***	7
NS-398										
0.1	198 ± 26***	2012 ± 115***	0.34 ± 0.02*	7.1 ± 0.5***	6	NT	NT	NT	NT	–
1	165 ± 32***	1154 ± 101***	0.22 ± 0.03***	4.1 ± 0.2***	7	815 ± 69***	ND	0.28 ± 0.03	41.5 ± 1.7	7
3	NT	NT	NT	NT	–	389 ± 56***	ND	0.50 ± 0.03***	67.2 ± 2.5***	8
10	ND	385 ± 74***	0.18 ± 0.03***	3.2 ± 0.1***	7	ND	ND	1.26 ± 0.10***	83.5 ± 6.3***	7

Each value represents the mean ± S.E.M. of *n* animals. *$P<0.05$, **$P<0.01$, ***$P<0.001$ vs. control group. ND = not detectable; NT = dose not tested.

multiple comparison test. The level of statistically significant difference was defined as $P < 0.05$.

3. Results

3.1. Effect of COX inhibitors on carrageenin pleurisy

We performed a series of experiments by treating rats with indomethacin and NS-398 according to the protocol carried out by Gilroy et al. [5] and obtained similar results (Table 1). Thus, we found that the pleural exudates of control rats, 2 h after carrageenin injection, contained small amounts of PGD_2 compared to PGE_2. These amounts were dose-dependently and significantly reduced by either indomethacin or NS-398. Conversely, in control rats at 48 h, the amounts of PGD_2 was increased by about 3.5 times, while PGE_2 was undetectable. Both indomethacin and NS-398 dose-dependently and significantly reduced PGD_2 levels in the 48 h exudates. We also observed that COX inhibitors suppressed exudate formation and cell migration at 2 h and increased them at 48 h. On no occasion did treatment with COX inhibitors modify the differential cell count ($>90\%$ polymorphonuclear leukocytes at 2 h and $>70\%$ mononuclear cells at 48 h). Moreover, as shown by Gilroy et al. [5], we found that injection of PGJ_2 (100 μg/kg) into the pleural cavity of rats, 24 h after carrageenin challenge, inhibited both exudate volume and cell migration by about 50% (Fig. 1). Furthermore, when PGJ_2 (100 μg/kg) was injected in rats previously treated with indomethacin (3.0 mg/kg) or with NS-398 (10 mg/kg), a complete reversion of the exacerbating effects of these COX inhibitors was observed. Similar results were obtained by using PGD_2 (data not shown).

3.2. NF-κB and HSF1 activation and hsp72 expression in rat carrageenin pleurisy

As shown in Fig. 2A, a retarded band of NF-κB/DNA complex was faintly detected in normal rats (i.e. animals which did not receive carrageenin or drugs). This band was greatly increased 2 h after carrageenin injection and decreased, but was still clearly detectable, 48 h later. The composition of the NF-κB complex activated by carrageenin was determined by competition and supershift experiments (Fig. 2A). The specificity of the NF-κB/DNA binding complex was

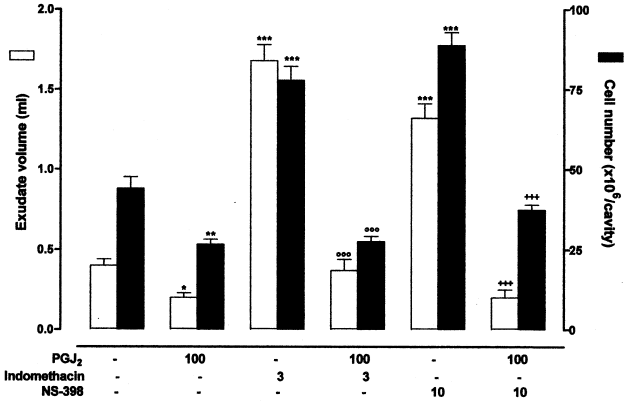

Fig. 1. Effects of PGJ_2, indomethacin and NS-398, given alone or in combination, on exudate volume (empty columns) and cell infiltration (filled columns) evaluated at 48 h. Each column represents the mean ± S.E.M. of 7–10 rats. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ vs. control group; $^{\circ\circ\circ}P < 0.001$ vs. indomethacin group; $^{+++}P < 0.001$ vs. NS-398 group.

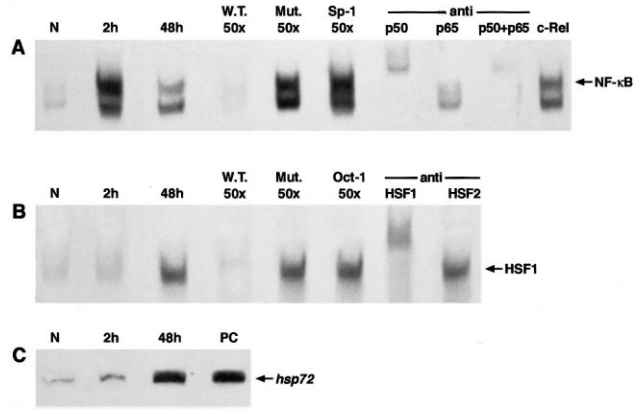

Fig. 2. Analysis of NF-κB/DNA binding activity (A), HSF1/DNA binding activity (B) and of *hsp72* protein expression (C) performed on pleural cells collected from normal rats (N) or from carrageenin-treated rats 2 h and 48 h after the challenge. A: Competition reaction in which nuclear extracts of inflammatory cells collected at 2 h were incubated with radiolabeled NF-κB probe in the absence or presence of identical but unlabeled oligonucleotide wild-type (W.T. 50×), mutated non-functional κB probe (Mut. 50×) or unlabeled oligonucleotide containing the consensus sequence for Sp-1 (Sp-1 50×) and supershift experiment in which the same nuclear extracts were incubated with antibodies against p50, p65, p50+p65 or c-Rel 30 min before incubation with radiolabeled NF-κB probe. B: Competition reaction in which nuclear extracts of inflammatory cells collected at 48 h were incubated with radiolabeled HSF probe in the absence or presence of identical but unlabeled oligonucleotide wild-type (W.T. 50×), mutated non-functional HSF probe (Mut. 50×) or unlabeled oligonucleotide containing the consensus sequence for Oct-1 (Oct-1 50×) and a supershift experiment in which the same nuclear extracts were incubated with antibodies against HSF1 or HSF2 30 min before incubation with radiolabeled HSF probe. C: *hsp72* evaluated by Western blot analysis. Recombinant human *hsp72* was used as a positive control (PC). Data illustrated in each panel are from a single experiment and are representative of three separate experiments.

demonstrated by the complete displacement of NF-κB/DNA binding in the presence of a 50-fold molar excess of unlabeled NF-κB probe in the competition reaction. In contrast, a 50-fold molar excess of unlabeled mutated NF-κB probe or Sp-1 oligonucleotide had no effect on this DNA binding activity. The composition of the NF-κB complex activated by carrageenin was determined by using specific antibodies against p50, p65 or p50+p65 and c-*Rel* subunits of NF-κB proteins. Addition of either anti-p50 or anti-p65 or anti-p50+anti-p65, but not anti-c-*Rel*, to the binding reaction resulted in a marked reduction of NF-κB band intensity and, in addition, the p50 antibody caused mobility retardation. EMSA performed on nuclear extracts from pleural cells exhibited a profile of HSF1/DNA binding activity opposite to that observed for NF-κB/DNA binding activity. Thus, HSF1/DNA binding activity was virtually undetectable in pleural cells from normal rats or from carrageenin-treated rats 2 h after injection, whereas maximum HSF1 activation was detected 48 h later (Fig. 2B). The enhanced activation of HSF1 observed at 48 h was associated with increased expression of *hsp72* compared to that observed in extracts from cells collected from either normal rats or carrageenin-treated rats 2 h after injection (Fig. 2C). Equal loading was confirmed by β-actin staining (data not shown).

In order to confirm the specificity of the HSF1/DNA com-

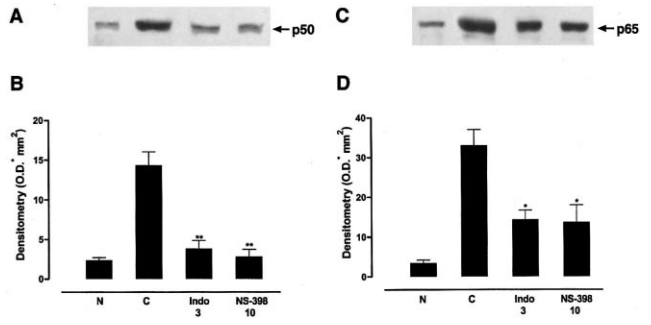

Fig. 3. Effects of indomethacin (Indo; 3 mg/kg) and NS-398 (10 mg/kg) on p50 and p65 nuclear translocation in inflammatory cells, collected 2 h after carrageenin injection, evaluated by Western blot (A,C) and densitometric analysis (B,D). N = cells from normal rats. Each immunoblot is from a single experiment and is representative of three separate experiments. Densitometry results are expressed as mean ± S.E.M. of three separate experiments. $*P < 0.05$, $**P < 0.01$ vs. control group (C).

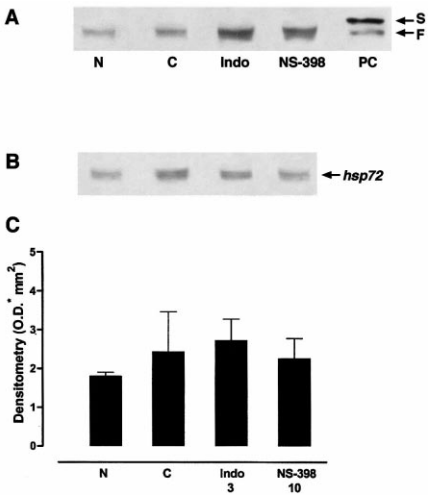

Fig. 4. Effects of indomethacin (Indo; 3 mg/kg) and NS-398 (10 mg/kg) on HSF1 activation and *hsp72* protein expression in inflammatory cells collected 2 h after carrageenin challenge. A: Western blot of HSF1 activation. Recombinant human HSF1 protein was used as a positive control (PC). HSF1 was immunoprecipitated by anti-HSF1 antibody and analyzed by immunoblotting using an anti-phosphoserine monoclonal antibody. The antibody used clearly detects the transcriptionally active, hyperphosphorylated form of HSF1 (S), as well as the inactive faster migrating isoform (F). B,C: *hsp72* protein expression evaluated respectively by Western blot and densitometric analysis. N = cells from normal rats; C = cells from control rats. Each immunoblot is from a single experiment and is representative of three separate experiments. Densitometry results are expressed as mean ± S.E.M. of three separate experiments.

plex activated by carrageenin we performed competition and supershift experiments (Fig. 2B). The specificity of the HSF1/DNA binding complex was demonstrated by the complete displacement of HSF1/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 this DNA binding activity. Furthermore, to establish which member of the HSF family was activated by carrageenin, nuclear cell extracts were preincubated with antibodies specific for either HSF1 or HSF2 and analyzed by EMSA. Addition of anti-HSF1 but not anti-HSF2 to the binding reaction resulted in both marked reduction of intensity and mobility retardation of the HSF band, thus indicating that carrageenin induces HSF1/DNA binding activity (Fig. 2B).

3.3. *Effect of COX inhibitors on p50 and p65 nuclear levels at 2 h*

In control animals (carrageenin alone), 2 h after carrageenin injection, high levels of both p50 and p65 NF-κB subunits were detected by immunoblotting analysis as compared to normal rats (Fig. 3A,C). As revealed by relative densitometric analysis (Fig. 3B,D), treatment of rats with indomethacin (3.0 mg/kg) or NS-398 (10 mg/kg) significantly inhibited both p50 (by 74 and 81%, respectively) and p65 (by 57 and 59%, respectively) nuclear levels.

3.4. *Effect of COX inhibitors on HSF1 activation and hsp72 expression at 2 h*

In control animals (carrageenin alone) 2 h after carrageenin injection, neither activation of HSF1 nor increased expression of *hsp72* was detected by immunoblotting analysis as compared to normal rats (Fig. 4A,B). In contrast, both non-steroidal anti-inflammatory drugs (NSAIDs) induced an incomplete stress response increasing the constitutively phosphorylated and transcriptionally inert isoform of HSF1, but not *hsp72* protein expression as demonstrated by immu-

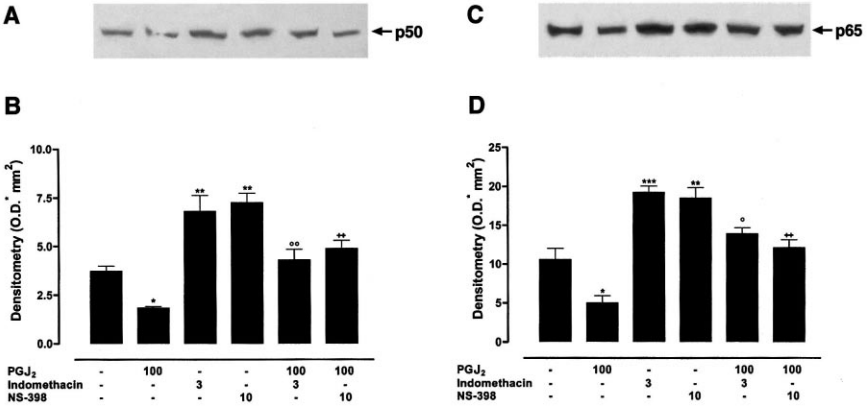

Fig. 5. Effects of PGJ_2, indomethacin and NS-398, given alone or in combination, on p50 and p65 nuclear translocation in inflammatory cells, collected 48 h after challenge, evaluated by Western blot (A,C) and densitometric analysis (B,D). Each immunoblot is from a single experiment and is representative of three separate experiments. Densitometry results are expressed as mean ± S.E.M. of three separate experiments. $*P < 0.05$, $**P < 0.01$ vs. control group; $°P < 0.05$, $°°P < 0.01$ vs. indomethacin group; $^{++}P < 0.01$ vs. NS-398 group.

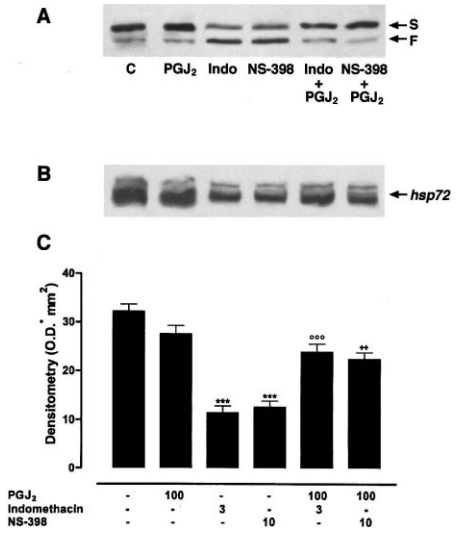

Fig. 6. Effects of PGJ_2, indomethacin and NS-398 given alone or in combination on HSF1 activation and *hsp72* protein expression in inflammatory cells collected 24 h after carrageenin challenge. A: Western blot of HSF1 activation. HSF1 was immunoprecipitated by anti-HSF1 antibody and analyzed by immunoblotting using an anti-phosphoserine monoclonal antibody. The antibody clearly detects the transcriptionally active, hyperphosphorylated form of HSF1 (S) as well as the inactive faster migrating HSF1 isoform (F). B,C: *hsp72* protein expression evaluated respectively by Western blot and densitometric analysis. Each immunoblot is from a single experiment and is representative of three separate experiments. Densitometry results are expressed as mean ± S.E.M. of three separate experiments. $^{***}P < 0.001$ vs. control group; $^{°°°}P < 0.001$ vs. indomethacin group; $^{++}P < 0.01$ vs. NS-398 group.

noprecipitation and Western blotting analysis (Fig. 4A,B). Equal loading was confirmed by β-actin staining (data not shown).

3.5. Effect of cyPGs and COX inhibitors on p50 and p65 nuclear levels at 48 h

As shown in Fig. 5A,C, low levels of both p50 and p65 NF-κB subunits were detected in nuclear extracts from control animals as compared to those measured at 2 h (Fig. 3A,C). Treatment of rats with PGJ_2 (100 μg/kg) significantly decreased both p50 and p65 nuclear levels by about 50%. In contrast, indomethacin and NS-398 increased both p50 and p65 band intensity by about 100% as compared to that of the control group. Coinjection of PGJ_2 with indomethacin or NS-398 almost completely reversed the NSAID-induced activation of p50 and p65 nuclear translocation. Similar results were also obtained with PGD_2 (data not shown).

3.6. Effect of cyPGs and COX inhibitors on HSF1 activation and hsp72 expression at 48 h

As shown in Fig. 6A,B, cells collected from control animals showed the formation of the hyperphosphorylated higher molecular weight isoform of HSF1, and a significant increase of *hsp72* protein expression as compared to cells collected at 2 h (Fig. 4A,B). Treatment of rats with PGJ_2 (100 μg/kg) only slightly modified HSF1 and *hsp72* protein expression as compared to the control group. In contrast, indomethacin (3.0 mg/kg) and NS-398 (10 mg/kg) induced an incomplete stress response increasing the band intensity of the constitutively phosphorylated, fast migrating HSF1 isoform but not *hsp72* protein expression, which, instead, was significantly reduced by 65% and by 63% respectively, as compared to the control group. This effect was due to incomplete phosphorylation of HSF1 induced by the two COX inhibitors [23], resulting in a reduction of the transcriptionally active, hyperphosphorylated HSF1 isoform and, consequently, of *hsp72* protein expression. Coinjection of PGJ_2 with indomethacin or NS-398 almost completely reversed the NSAID-induced effect on HSF1 activation and *hsp72* expression (Fig. 6A,B). Equal loading was confirmed by β-actin staining (data not shown). Similar results were also obtained with PGD_2 (data not shown).

4. Discussion

We have recently shown that the inhibition of HSF1 activation reduced *hsp72* expression and exacerbated carrageenin-induced pleurisy, suggesting a relevant role for the HSF1/*hsp72* pathway as an endogenous anti-inflammatory system [13]. In this study we demonstrate that the HSF1/*hsp72* pathway is triggered by cyPGs and protects the organism from the deleterious effect of protracted activation of the inflammatory response. In fact, in rat carrageenin-induced pleurisy, the pleural exudate of the early phase (2 h) contained low amounts of PGD_2 compared to PGE_2 amounts (this paper and [5]) and the inflammatory cells showed constitutive levels of both HSF1 and *hsp72* and activation of NF-κB. However, in the exudate collected at the resolution phase (48 h), PGD_2 levels were increased by about 3.5-fold whereas PGE_2 was undetectable (this paper and [5]), and the cells showed activation of HSF1, increased expression of *hsp72* and inhibition of NF-κB. Treatment of rats with both the COX-2 selective inhibitor NS-398 and the dual COX-1/COX-2 inhibitor indomethacin suppressed the pleurisy at 2 h, but significantly exacerbated it at 48 h confirming previous findings by Gilroy et al. [5]. Exacerbated reaction was associated with reduced PGD_2 levels, decreased HSF1-induced *hsp72* expression and, more interestingly, increased NF-κB activation. Replacement of cyPGs by treating rats with PGJ_2 or PGD_2 reversed the exacerbating effects of COX inhibitors leading to resolution of inflammation. cyPGs are a class of pharmacologically active compounds, derivatives of PGD_2, with intriguing properties. In fact, they are characterized by the ability to activate the inducible phosphorylated form of HSF1, which in turn activates *hsp72* synthesis for extended periods (12–24 h) [9,10]. Inducible phosphorylation appears to be essential for transcriptional activation since chemicals such as salicylates and the NSAIDs aspirin and indomethacin cause HSF trimerization, nuclear translocation and binding to the heat shock element of the endogenous *hsp70* gene. However, they are unable to activate HSF phosphorylation, thus inducing a transcriptionally inert DNA binding trimeric state, which is unable to trigger the expression of *hsp* genes [11,19]. Cyclopentenone prostaglandins have also been shown to inhibit the activation of NF-κB [20,21], an inducible transcription factor responsible for the early inflammatory response and activation of the immune system [22,23]. We have recently demonstrated that one of the earliest molecular event following challenge of rats with carrageenin is the activation of NF-κB which peaks at 24 h and decreases along with the resolution of the inflammatory reaction [24]. Herein, our results suggest different roles for HSF1 and NF-κB in the gene reprogramming during inflammation. In fact, the present study demonstrates that in the early phase of inflammation, when polymorphonuclear

cells dominate, NF-κB plays a relevant role in pro-inflammatory gene programming whereas, in the late, mononuclear cells-dominated phase it is essentially HSF1 that drives the reaction to resolution by selectively inducing the expression of protective *hsp*. Thus it is possible to hypothesize that cyPGs, synthesized in large quantities by COX-2 during the late phase of carrageenin-induced pleurisy, drive the inflammation to resolution acting through a double mechanism. On the one hand cyPGs activate HSF1, resulting in increased expression of cytoprotective *hsp72* synthesis, and on the other hand, they directly inhibit NF-κB activation resulting in repression of pro-inflammatory gene transcription. This dual action of cyPGs suggests that potential novel therapeutic strategies may rely upon the simultaneous activation of cytoprotective genes and downregulation of inflammatory genes, uncovering a new scenario in which the cytoprotective effect of the heat shock response is amplified by rendering the cells unresponsive to pro-inflammatory signals. Another interesting issue raised by our study is the dual effect exhibited by indomethacin and by the selective COX-2 inhibitor NS-398 towards NF-κB activation at the different phase of the inflammatory reaction. In fact, when these drugs were administered to rats at the early phase of carrageenin pleurisy they exhibited the expected anti-inflammatory effects and significantly reduced NF-κB activation. However, when these agents were administered to rats 24 h after carrageenin injection the pleurisy was exacerbated, as shown by Gilroy et al. [5]. Moreover, in human cells, salicylate as well as other NSAIDs were found to induce HSF1 activation which was not associated with an increased transcription of heat shock genes [18,25]. In fact, also in our experimental model, both NSAIDs used, irrespective of their selectivity towards COX isoforms, triggered only an incomplete activation of HSF1, not accompanied by hyperphosphorylation, and thus transcriptionally inert where expression of *hsp72* was not detected. In conclusion, this study shows that cyPGs may act as anti-inflammatory mediators by inducing the HSF1/*hsp72* pathway and by inhibiting NF-κB activation. Furthermore, we suggest that the use of agents able to trigger simultaneously the activation of HSF1, which dictates the synthesis of protective chaperones, and the inhibition of NF-κB, which starts the synthesis of relevant pro-inflammatory mediators, may represent a novel therapeutic approach in the treatment of inflammatory diseases.

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References

[1] Tomlinson, A., Appleton, I., Moore, A.R., Gilroy, D.W., Willis, D., Mitchell, J.A. and Willoughby, D.A. (1994) Br. J. Pharmacol. 113, 693–698.
[2] Willis, D., Moore, A.R., Frederick, R. and Willoughby, D.A. (1996) Nature Med. 2, 87–90.
[3] DeWitt, D.L. (1991) Biochim. Biophys. Acta 1083, 121–134.
[4] Vane, J.R. (1994) Nature 367, 215–216.
[5] Gilroy, D.W., Colville-Nash, P.R., Willis, D., Chivers, J., Paul-Clark, M.J. and Willoughby, D.A. (1999) Nature Med. 5, 698–701.
[6] Li, A.C., Willson, T.M., Kelly, C.J. and Glass, C.K. (1998) Nature 391, 79–82.
[7] Jiang, C., Ting, T.A. and Seed, B. (1998) Nature 391, 82–86.
[8] Rossi, A., Elia, G. and Santoro, M.G. (1997) Proc. Natl. Acad. Sci. USA 94, 746–7508.
[9] Santoro, M.G., Garaci, E. and Amici, C. (1989) Proc. Natl. Acad. Sci. USA 86, 8407–8411.
[10] Amici, C., Sistonen, L., Santoro, M.G. and Morimoto, R.I. (1992) Proc. Natl. Acad. Sci. USA 89, 6227–6231.
[11] Cotto, J.J., Kline, M. and Morimoto, R.I. (1996) J. Biol. Chem. 271, 3355–3358.
[12] Lindquist, S. and Craig, E.A. (1988) Annu. Rev. Genet. 22, 631–677.
[13] Ianaro, A., Ialenti, A., Maffia, P., Pisano, B. and Di Rosa, M. (2001) FEBS Lett. 499, 239–244.
[14] D'Acquisto, F., Ialenti, A., Ianaro, A. and Carnuccio, R. (1999) Naynyn-Schmiedeberg's Arch. Pharmacol. 360, 670–675.
[15] Sarge, K.D., Zimarino, V., Holm, K., Wu, C. and Morimoto, R.I. (1992) Genes Dev. 5, 1902–1911.
[16] Tacchini, L., Radice, L., Pogliaghi, G. and Bernelli-Zazzera, A. (1997) Hepatology 26, 186–191.
[17] Ethridge, R.T., Hellmich, M.R., DuBois, R.N. and Evers, B.M. (1998) Gastroenterology 115, 1454–1463.
[18] Schett, G., Redlich, K., Xu, Q., Bizan, P., Groger, M., Tohidast-Akrad, M., Kiener, H., Smolen, J. and Steiner, G. (1998) J. Clin. Invest. 102, 302–311.
[19] Lee, B.S., Chen, J., Angelidis, C., Jurivich, D.A. and Morimoto, R.I. (1995) Proc. Natl. Acad. Sci. USA 92, 7207–7211.
[20] Rossi, A., Kapahi, P., Natoli, G., Takahashi, T., Chen, Y., Karin, M. and Santoro, M.G. (2000) Nature 403, 103–108.
[21] Straus, D.S., Pascual, G., Li, M., Welch, J.S., Ricote, M., Hsiang, C.H., Sengchanthalangsy, L.L., Ghosh, G. and Glass, C.K. (2000) Proc. Natl. Acad. Sci. USA 97, 4844–4849.
[22] Baldwin, A.S. (1996) Annu. Rev. Immunol. 14, 649–683.
[23] Barnes, P.J. and Karin, M. (1997) New Engl. J. Med. 336, 1066–1071.
[24] D'Acquisto, F., Ianaro, A., Ialenti, A., Iuvone, T., Colantuoni, V. and Carnuccio, R. (1999) Eur. J. Pharmacol. 369, 233–236.
[25] Jurivich, D.A., Sistonen, L., Kroes, R.A. and Morimoto, R.I. (1992) Science 255, 1243–1245.