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Inhibition of cyclooxygenase-2 gene expression by the heat shock response in J774 murine macrophages

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Abstract

The heat shock response is a highly conserved mechanism of protection elicited in the cell by various kinds of stimuli, such as heat, sodium arsenite, oxidants and inflammation. Among the mechanisms potentially involved in mediating the protective effects of *hsp*, one of the most investigated is the inhibition of pro-inflammatory gene expression such as inducible nitric oxide synthase (iNOS) and inflammatory cytokines. Nevertheless, data about the effects of heat shock response on cyclooxygenase-2 expression in activated macrophages are so far not available in literature. The aim of this study was to investigate the changes in cyclooxygenase-2 expression following lipopolysaccharide stimulation of heat shocked J774 murine macrophages. We found, by Western blotting analysis and reverse transcription–polymerase chain reaction analysis (RT-PCR), that the lipopolysaccharide-induced cyclooxygenase-2 gene expression was reduced in heat shocked cells. Such a reduction was associated to activation of heat shock factor, increased levels of heat shock protein 72 and inhibition of lipopolysaccharide-induced nuclear factor-κB binding activity. These data suggest that the heat shock response inhibits cyclooxygenase-2 gene expression at transcriptional level, i.e. by preventing the activation of nuclear factor-κB, and provide additional information about mechanism(s) underlying the anti-inflammatory effect of the heat shock proteins.

Keywords: Cyclooxygenase-2; Heat shock factor; Heat shock protein 72; Nuclear factor-κB; Transcription factor

1. Introduction

The heat shock response is a highly conserved mechanism of protection elicited in the cell by several stimuli, such as heat, sodium arsenite, oxidants and inflammation (Lindquist, 1986). This mechanism is brought about by the activation of heat shock factor (HSF) that leads to the expression of a specific set of proteins (heat shock proteins, *hsp*) and the transient repression of non-heat shock protein gene expression (Wong et al., 1997). The *hsp* are known to function as molecular chaperones during protein folding/assembly and membrane translocation and to prevent aggregation of misfolded polypeptide chains in cells (Han et al., 2001). 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, 2003; Van Molle et al., 2002). Among the mechanisms potentially involved in mediating the protective effects of *hsp*, one of the most investigated is the inhibition of pro-inflammatory genes expression. Recent findings, showing that HSF1, the most involved member of HSF family (from HSF1 to HSF4) in heat shock response, is a

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repressor of pro-inflammatory cytokines gene transcription, suggest a role for the transcription factor as an antagonist of inflammatory process (Cahill et al., 1996). In fact, it has been shown that the heat shock response inhibits the release of interleukin-6 by fibroblast (Simon et al., 1995), and the production of both tumor necrosis factor-α (TNF-α) and interleukin-1β by mononuclear cell at a transcriptional (Snyder et al., 1992; Schmidt and Abdulla, 1988) and post-translational (Ribeiro et al., 1994) level. Moreover, it has been demonstrated that the heat shock response inhibits inducible nitric oxide synthase (iNOS) gene expression stimulated by either cytokines or lipopolysaccharide (Wong et al., 1995; Feinstein et al., 1996; Scarim et al., 1998; Hauser et al., 2001). This inhibitory effect was found to be associated with the expression of *hsp70* (Wong et al., 1997). Furthermore, a role for *hsp70* in heat shock-induced iNOS suppression is supported by the demonstration that transfection of glial cells with human *hsp70* cDNA partially replicated the heat shock effects, and that rat fibroblasts stably expressing human *hsp70* did not express iNOS in response to lipopolysaccharide plus cytokines (Feinstein et al., 1996).

Another pro-inflammatory gene, whose expression seems to be affected by the heat shock response, is that coding for the inducible isoform of cyclooxygenase (cyclooxygenase-2) expressed in response to a variety of inflammatory stimuli, cytokines and mitogens (Colville-Nash and Gilroy, 2000). In fact, it has been observed a reduced increase of cyclooxygenase-2 mRNA in the skin of mice exposed to heat shock before lipopolysaccharide challenge (Suganuma et al., 2002). Nevertheless, data about the effects of heat shock response on cyclooxygenase-2 expression in activated macrophages are so far not available in literature.

In the present study, we have investigated the cyclooxygenase-2 gene expression following lipopolysaccharide stimulation of heat shocked murine macrophages. We show that the heat shock response reduced the lipopolysaccharide-induced expression of cyclooxygenase-2, an effect that was associated to activation of HSF1, increased levels of *hsp72* and inhibition of NF-κB binding activity.

2. Methods

2.1. Cell culture

The murine monocyte/macrophage cell line J774 was from the European Collection of Animal Cell Cultures (Salisbury, UK). J774 cells were grown in Dulbecco's modified Eagle's medium and cultured at 37 °C in humidified 5% CO_2/95% air. Culture medium was supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 25 mM HEPES, 100 U ml^{-1} penicillin, 100 μg ml^{-1} streptomycin and 5 mM sodium pyruvate. The cells were plated onto 24-well culture plates at a density of 2.5×10^5/ml/well or on 100-mm tissue culture dishes at a density of 1×10^7/dish and allowed to adhere for 2 h. Thereafter, the medium was replaced with fresh medium. Heat shock was induced by incubating cells at 42 °C for 1 h. Cells were subsequently activated by 0.1 μg ml^{-1} lipopolysaccharide from *E. coli*.

In preliminary experiments, we established that cell viability (>95%) was not affected by heat treatment (data not shown).

2.2. RNA extraction and polymerase chain reaction (PCR) amplification of reverse-transcribed mRNA

Cyclooxygenase-2 mRNA expression in J774 cell line was quantified by PCR amplification of reverse-transcribed mRNA. Total RNA was extracted with a RNeasy Mini Kit according to the manufacturer's instructions. A one-step RT-PCR kit (Qiagen, Hilden, Germany) was used with the following components: 10 μl of RT-PCR buffer containing Tris–HCl, KCl $(NH_4)_2SO_4$ and 2.5 mM $MgCl_2$; 2 μl of deoxynucleotide mixture containing 400 μM of dATP, dCTP, dGTP and dTTP, respectively; 2 μl of enzyme mixture containing Omniscript reverse transcriptase, Sensiscript reverse transcriptase and HotStar Taq DNA polymerase; 1 μl of RNase inhibitor (40 U); 3 μl of each random primer (10 pmol μl^{-1}); and 1 μg of substrate RNA. RNase-free water was added to each reaction to a final volume of 50 μl. Primers utilised were as follows: cyclooxygenase-2 sense 5′-CCGGGTTGCTGGGGGAAGA-3′, cyclooxygenase-2 antisense 5′-GTGGCTGTTTTGGTAGGCTGTGGA-3′, β-actin sense 5′ ATGAAGATCCTGACCGAGCGT3′, β-actin antisense 5′ AACGCAGCTCAGTAACAGTCCG3′ . The tubes were placed in the thermocycler at 50 °C for 30 min for reverse transcription, 95 °C for 1.5 min for initial denaturation, followed by 29 cycles of the following sequential steps: 30 s at 94 °C (denaturation), 56 °C for both cyclooxygenase-2 and for β-actin (annealing), respectively, and 45 s at 72 °C (extension). The final extension was performed at 72 °C for 10 min: 10 μl of the PCR-amplified mixture was subjected to electrophoresis on a 2% agarose gel and DNA was visualised by ethidium bromide staining. Location of the predicted PCR products (base pairs) was confirmed by using a 100-base pair ladder as standard size maker.

2.3. Preparation of cell extracts

All the extraction procedures were performed on ice with ice-cold reagents. Unstimulated or lipopolysaccharide-stimulated (0.1 μg/ml) J774 macrophages, subjected or not to heat shock, were washed twice in phosphate-buffered saline (PBS) and centrifuged at 800×*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×*g* at 4 °C for 5 min, the protein concentration in the supernatant was determined by the

Bio-Rad protein assay kit and then it was aliquoted and stored at −80 °C.

2.4. Preparation of nuclear extracts

All the extraction procedures were performed on ice with ice-cold reagents. Unstimulated or lipopolysaccharide-stimulated (0.1 μg/ml) J774 macrophages, subjected or not to heat shock, were washed twice with ice-cold PBS and centrifuged at 1500×*g* for 10 min at 4 °C. The cell pellet was resuspended in one packed cell volume of lysis buffer and incubated on ice for 5 min with occasional vortexing. After centrifugation at 1500×*g* at 4 °C for 5 min, 1 cell pellet volume of extraction buffer was added to the nuclear pellet and incubated on ice for 15 min with occasional vortexing. Nuclear proteins were isolated by centrifugation at 13,000×*g* for 15 min, the supernatant was aliquoted and stored at −80 °C. Protein concentration was determined by the Bio-Rad protein assay kit.

2.5. 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 idealised 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 labelled with γ-[^{32}P]ATP and gel mobility assay was performed as described (Ianaro et al., 2001b). Aliquots of cell extracts (10 μg protein for each sample) were incubated for 30 min at room temperature with radiolabeled oligonucleotides (2.5–5.0×10^4 cpm) in 20 μl reaction buffer containing 2 μg poly dI-dC, 10 mM Tris–HCl (pH 7.5), 100 mM NaCl, 1 mM EDTA, 1 mM DTT, 1 mg ml^{-1} 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. The specificity of the DNA/protein binding for HSF was determined 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 or HSF2 proteins and to p50, p65 or c-Rel subunits of NF-κB complex were added to the reaction mixture 30 min before the addition of radiolabeled probes. Protein-nucleic acid complexes were resolved by electrophoresis on 4% non-denaturing polyacrylamide gel in 0.5× Tris-borate/EDTA at 150 V for 2 h at 4 °C. The gel was dried and autoradiographed with intensifying screen at −80 °C for 20 h. Subsequently, the relative bands were quantified by densitometric scanning of the X-ray films with GS-700 Imaging Densitometer (Bio-Rad, Milan, Italy) and a computer program (Molecular Analyst, IBM, Milan, Italy).

2.6. Western blot analysis

Immunoblotting analysis of cyclooxygenase-2, *hsp72* and β-actin proteins was performed on whole cell extracts. Equivalent amounts of protein (40 μg) from each sample were electrophoresed in an 8% discontinuous polyacrylamide minigel. The proteins were transferred onto nitrocellulose membranes, according to the manufacturer's instructions (Bio-Rad). The membranes were saturated by incubation at 4 °C overnight with 10% non-fat dry milk in PBS-0.1% Triton X-100 and then incubated with anti-*hsp72* rabbit antibody (1:10,000), anti-cyclooxygenase-2 (1:250) or anti-β-actin (1:5000) mouse antibodies for 2 h at room temperature. The membranes were washed three times with 0.1% Tween 20 in PBS and then incubated with anti-rabbit (1:10,000) or anti-mouse (1:2000) immunoglobulins coupled to peroxidase. The immune complexes were visualised by the enhanced chemiluminescence method (Amersham, Cologno Monzese, Italy). Subsequently, the relative expression of *hsp72*, cyclooxygenase-2 and β-actin was quantified by densitometric scanning of the X-ray films with GS-700 Imaging Densitometer (Bio-Rad) and a computer program (Molecular Analyst, IBM).

2.7. Prostaglandin E_2 assay

The accumulation of prostaglandin E_2 in the cell culture medium was measured, without prior extraction or purification, by EIA according to the manufacturer's instruction. Results are expressed as pg/ml of prostaglandin E_2 and represent the mean±S.E.M. of *n* experiments runned in triplicate.

2.8. Reagents

Fetal bovine serum and Dulbecco's modified Eagles medium were from BioWhittaker (Heidelberg, Germany). Twenty-four-well culture plates and 100-mm tissue culture dishes were from Falcon (Meylan, France). lipopolysaccharide from *E. coli* was from Fluka (Milan, Italy). Rneasy Mini Kit and random primers were from Quiagen (Hilden, Germany). Bio-rad protein assay kit and non-fat dry milk were from Bio-Rad. Oligonucleotides were synthesised by Roche Biomol (Milan, Italy). PBS was from Celbio (Milan, Italy). DL-dithiothreitol, phenylmethylsulfonilfluoride, soybean trypsin inhibitor, pepstatin A, leupeptin and benzamidine were from Calbiochem (Milan, Italy). ^{32}P-γ-ATP was from ICN Biomedicals (Milan, Italy). Poly dI-dC was from Boehringer-Mannheim (Milan, Italy). Anti-*hsp72* and anti HSF1 antibody was from StressGen (Victoria BC, Canada), anti HSF2 was from LabVision (Newmarket Suffolk, UK), anti p50, p65 and c-Rel was from Santa Cruz Biotechnology (Santa Cruz, CA), while anti-cyclooxygenase-2 was from Transduction Laboratories (Milan, Italy). EIA kit for postaglandin E_2 was from Cayman (Milan, Italy). All other reagents were from Sigma (Milan, Italy).

2.9. Statistical analysis

Values are expressed as the mean±S.E.M. of *n* experiments run in triplicate. Comparisons were calculated by one-way analysis of variance and Bonferroni-corrected *P*-value for multiple comparisons. The level of statistically significant difference was defined as $P<0.05$.

3. Results

3.1. Heat shock induces HSF1 activation and hsp72 expression in J774 macrophages

To detect HSF/DNA binding activity, an EMSA was performed on whole extracts from cells incubated for 1 h at 42 °C and harvested 2 h after lipopolysaccharide challenge. As shown in Fig. 1A, a retarded band of DNA-protein complex was clearly detected in heat-treated cells either unstimulated or lipopolysaccharide-challenged while it was faintly detected in cells not submitted to heat shock.

In order to confirm the specificity of the HSF/DNA complex, we performed competition experiments (Fig. 1B). The specificity of 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 this DNA-binding activity (Fig. 1B). Furthermore, to establish which member of the HSF family was activated by heat, nuclear extracts were preincubated with antibodies specific for either HSF1 or HSF2 and analysed by EMSA. Addition of anti-HSF1 but not anti-HSF2 to the binding reaction resulted in mobility retardation of the HSF band, thus indicating that heat activates HSF type 1 (Fig. 1B).

Western blot analysis showed a substantial increase of *hsp72* protein levels in extracts from heat-treated cells harvested 4 h after lipopolysaccharide challenge compared to that observed in extracts from control cells (Fig. 1C). Lipopolysaccharide treatment caused an increase in *hsp72* expression both at 37 and 42 °C (Fig. 1C). β-Actin Western

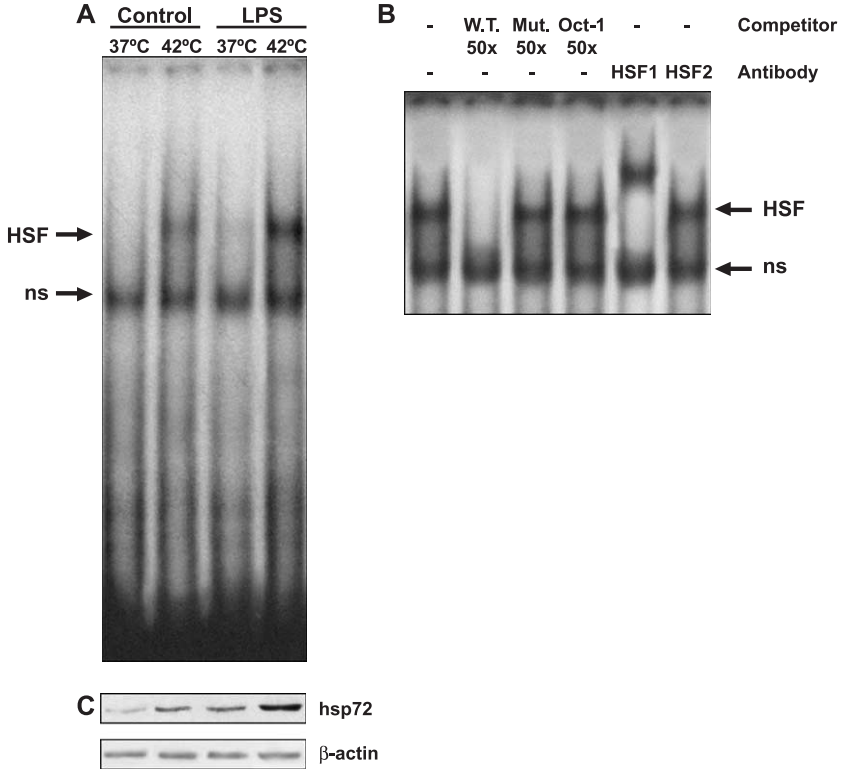

Fig. 1. (A) Effect of heat and lipopolysaccharide stimulation on HSF1/DNA binding activity. EMSA was performed on whole extracts from heat shocked or not cells harvested 2 h after lipopolysaccharide challenge. Data are representative of three separate experiments. (B) Characterisation of lipopolysaccharide-induced HSF1/DNA binding activity. In competition reaction, whole extracts of activated J774 macrophages were incubated with radiolabeled HSF probe in the absence or presence of 50-fold molar excess of unlabeled HSF probe (W.T. 50×), mutated non-functional HSF probe (Mut. 50×) or oligonucleotide containing the consensus sequence for Oct-1 (Oct-1 50×). In supershift experiments, whole extracts were incubated with antibodies against HSF1 or HSF2 30 min before incubation with the radiolabeled HSF probe. (C) Effect of heat and lipopolysaccharide stimulation on *hsp72* expression. Western blot analysis was performed on whole extracts from heat shocked or not cells harvested 4 h after lipopolysaccharide challenge. Equal loading was ensured by β-actin staining. Data are representative of three separate experiments.

blot analysis was performed to ensure equal sample loading (Fig. 1C).

3.2. Heat shock inhibits cyclooxygenase-2 gene expression and prostaglandin E_2 production

To determine the effect of the heat shock response on cyclooxygenase-2 gene expression, we performed a PCR amplification of reverse-transcribed cyclooxygenase-2 mRNA. Heat-shock induced a very slight increase of cyclooxygenase-2 mRNA expression not sufficient to induce cyclooxygenase-2 expression and prostaglandine E_2 production (Fig. 2). More interesting lipopolysaccharide-induced cyclooxygenase-2 mRNA expression, evaluated 6 h after lipopolysaccharide stimulation, was inhibited in heat shocked cells (Fig. 2A).

In immunoblotting experiments performed on whole extracts from control cells, cyclooxygenase-2 expression was not detectable, whereas stimulation of the cells with lipopolysaccharide for 8 h resulted in a strong increase in cyclooxygenase-2 protein expression (Fig. 2B). Incubation of the cells at 42 °C for 1 h before lipopolysaccharide treatment significantly inhibited the lipopolysaccharide-induced cyclooxygenase-2 protein expression, as shown by a marked reduction of cyclooxygenase-2 band intensity and by relative densitometric analysis (Fig. 2B). β-Actin Western blot analysis was performed to ensure equal sample loading (Fig. 2B).

Unstimulated J774 macrophages generated in 24 h 37±2 pg/ml prostaglandin E_2 (n=5) and 35±3 pg/ml prostaglandin E_2 (n=7) when cultured at 37 and 42 °C, respectively. Stimulation of the cells with bacterial lipopolysaccharide produced a massive and significant (P<0.001) increase in the production of this prostanoid (2150±190 pg/ml, n=6). Exposure of J774 to heat shock 1 h prior to lipopolysaccharide challenge significantly inhibited prostaglandin E_2 generation by 17% (P<0.01, n=7) (Fig. 2C).

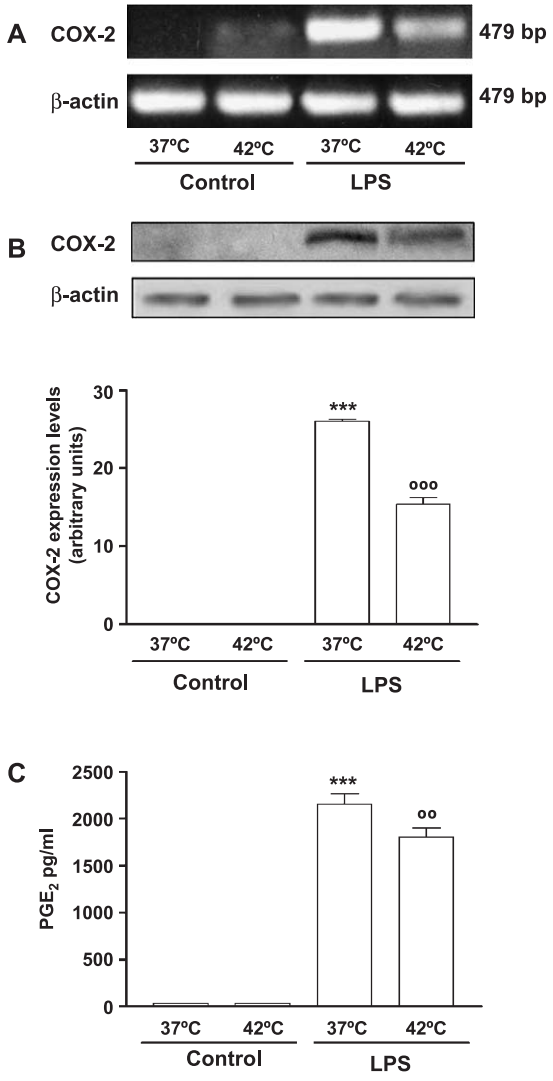

Fig. 2. (A) Effect of heat shock on lipopolysaccharide-induced cyclooxygenase-2 mRNA expression. PCR amplification of reverse-transcribed mRNA was performed on total RNA isolated from heat shocked or not cells harvested 6 h after lipopolysaccharide challenge. Parallel amplification of mouse housekeeping gene β-actin was performed as internal control. Data are representative of three separate experiments. (B) Effect of heat shock on lipopolysaccharide-induced cyclooxygenase-2 expression Western blot analysis (upper panel) was performed on whole extracts from heat shocked or not cells harvested 8 h after lipopolysaccharide challenge. Equal loading was ensured by β-actin staining. Data are representative of three separate experiments. Densitometric analysis of COX-2 protein expression levels is shown in lower panel. Data are expressed as mean±S.E.M. of three experiments. ***P<0.001 vs. control 37 °C; °°°P<0.001 vs. lipopolysaccharide 37 °C. (C) Effect of heat shock on lipopolysaccharide-induced prostaglandin E_2 production by J774 macrophages. Prostaglandin E_2 production was evaluated by EIA. Data are expressed as mean±S.E.M. of three experiments runned in triplicate. ***P<0.001 vs. control 37 °C; °°P<0.01 vs. lipopolysaccharide 37 °C.

3.3. Heat shock inhibits NF-κB activation in J774 macrophages

The transcription factor NF-κB plays an important role in the control of lipopolysaccharide-induced transcription of cyclooxygenase-2 gene. Treatment of J774 macrophages with lipopolysaccharide for 2 h resulted in activation of NF-κB complex evaluated by EMSA in cell nuclear extracts. Induction of heat shock response 1 h prior lipopolysaccharide challenge greatly decreased the intensity of the NF-κB/DNA complex (Fig. 3A, upper panel) as also shown by relative densitometric analysis (Fig. 3A, lower panel).

The specificity of NF-κB/DNA binding complex was demonstrated by the complete displacement of the 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 (Fig. 3B). The composition of the NF-κB complex activated by lipopolysaccharide was determined by performing supershift experiments using specific antibodies against p50, p65 and c-Rel subunits of NF-κB complex (Fig. 3B). Addition of either anti-p50 or

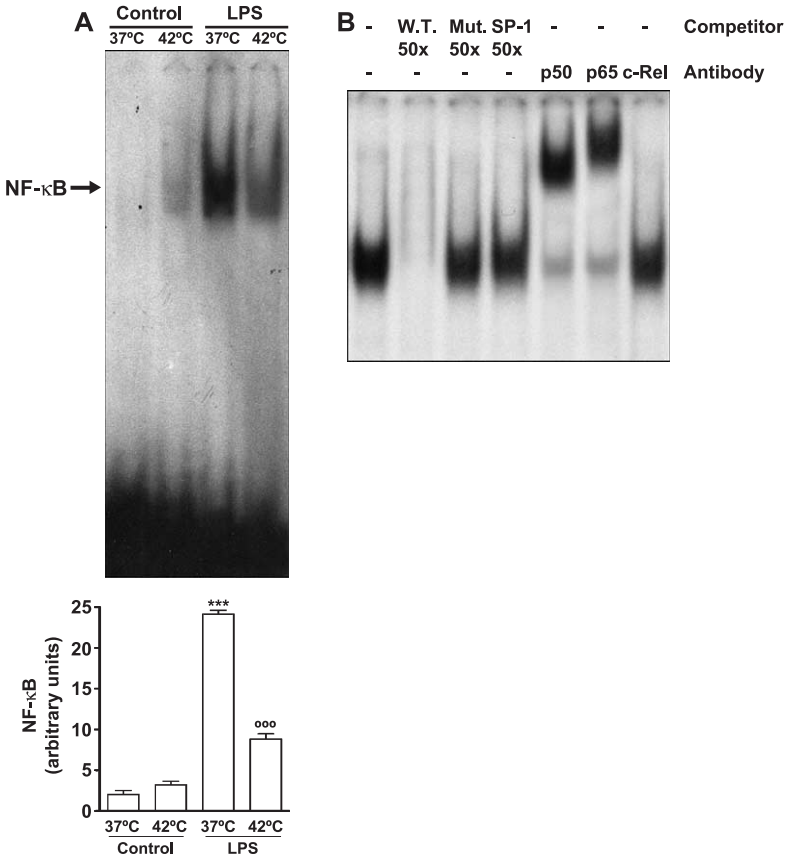

Fig. 3. (A) Effect of heat shock on lipopolysaccharide-induced NF-κB/DNA binding activity. EMSA was performed on nuclear extracts from heat shocked or not cells harvested 2 h after lipopolysaccharide challenge (upper panel). Data are representative of three separate experiments. Densitometric analysis of NF-κB DNA binding activity is shown in lower panel. Data are expressed as mean±S.E.M. of three experiments. ***$P<0.001$ vs. control 37 °C; °°°$P<0.001$ vs. lipopolysaccharide 37 °C. (B) Characterisation of lipopolysaccharide-induced NF-κB/DNA binding activity. In competition, reaction nuclear extracts of activated J774 macrophages were incubated with radiolabeled NF-κB probe in absence or presence of identical but unlabeled oligonucleotide (W.T. 50×), mutated non-functional κB probe (Mut. 50×) or unlabeled oligonucleotide containing the consensus sequence for Sp-1 (Sp-1 50×). In supershift experiments, nuclear extracts were incubated with antibodies against p65, p50 or c-Rel 30 min before incubation with the radiolabeled NF-κB probe.

anti-p65, but not anti-c-Rel caused both retardation of mobility and decrease in intensity of NF-κB band.

4. Discussion

In the present study, we investigated the lipopolysaccharide-induced cyclooxygenase-2 expression in heat shocked murine macrophages. We have shown that the heat shock response reduces the lipopolysaccharide-induced expression of cyclooxygenase-2 and that this inhibition is associated to increased levels of *hsp72*. These results provide additional information about the mechanism(s) underlying the anti-inflammatory effect of the heat shock proteins. Activation of the host immune system by Gram-negative bacteria can be reproduced in vitro by incubating cells with lipopolysaccharide and/or pro-inflammatory cytokines. Macrophages actively participate in the onset of inflammation and immune system activation by releasing cytokines, growth factors and lipid mediators such as prostaglandins and leukotrienes. Elevated prostaglandin levels are associated with the activation of immune system and both acute and chronic inflammation (DeWitt, 1991; Smith and Marnett, 1991). The synthesis of prostaglandins is dependent on the activity of cyclooxygenase enzyme of which two isoforms (cyclooxygenase-1 and cyclooxygenase-2) have been identified. cyclooxygenase-1, that is costitutively expressed in most tissue producing low levels of prostaglandins for physiological housekeeping functions, and cyclooxygenase-2, the inducible isoform of cyclooxygenase, expressed in response to a variety of inflammatory stimuli, cytokines and mitogens (Colville-Nash and Gilroy, 2000). In addition to its pro-inflammatory effects, cyclooxygenase-2 has been shown to have anti-inflammatory properties. Thus, Gilroy et al. (1999) and Ianaro et al. (2001b) showed, in a rat carrageenin-induced pleurisy model, that cyclooxygenase-2 protein expression peaked at 2 h and this peak was associated with maximal prostaglandin E_2 synthesis and marked inflammation. However, at a later stage of the inflammatory reaction (24 h) cyclooxygenase-2 expression exhibited a second peak that was associated

with reduced prostaglandin E_2 synthesis, increased levels of prostaglandin D_2 and prostaglandin J_2 and decreased inflammation. These data indicate that the prostaglandin E_2 type prostaglandins are associated with pro-inflammatory mechanisms, whereas the prostaglandin D_2 and prostaglandin J_2 forms are involved in the suppression of inflammation.

In our study, we demonstrate that activation of the heat shock response in J774 murine macrophages down-regulates the expression of cyclooxygenase-2 and inhibits the synthesis of pro-inflammatory prostaglandin E_2 both induced by lipopolysaccharide stimulation. These effects are associated to heat shock factor activation, increased levels of *hsp72* and inhibition of NF-κB binding activity.

The transcription factor NF-κB has been shown to control the lipopolysaccharide-induced transcription of cyclooxygenase-2 gene in macrophages (Wu et al., 2003) and up-regulate cyclooxygenase-2 expression (Dumais et al., 1998; Castrillo et al., 2000; Rossi et al., 2000; Straus et al., 2000). Interestingly, since in a variety of experimental models (Rossi et al., 1997, 1998; Morimoto and Santoro, 1998; Santoro, 2000), the inhibition of NF-κB has been shown to be associated with the activation of HSF1, it has been hypothesised that the same stimuli (e.g. lipopolysaccharide, cytokines, etc.) that activate one of these transcription factors also lead to the inhibition of the other one and vice-versa establishing a sort of 'yin-yang' mechanism, which protects cells from excess of stimulation and contributes to the regulation of cell homeostasis. Consistent with this hypothesis, we have previously demonstrated that oligodeoxynucleotides decoy for HSF1 inhibit *hsp* genes expression resulting in exacerbation of the inflammatory process (Ianaro et al., 2003). Therefore, *hsp* genes may be considered as anti-inflammatory or "therapeutic" genes, and their selective in vivo transactivation, by appropriate pharmacological agents, may lead to the remission of the inflammatory reaction. In this study we demonstrate that an opposite regulation exists between HSF1/*hsp72* and NF-κB pathways and that this regulatory system modulates the expression of a key pro-inflammatory enzyme such as cyclooxygenase-2. In fact, in this study, we show that the activation of heat shock response in murine macrophages inhibits lipopolysaccharide-induced cyclooxygenase-2 gene expression and this effect is associated to NF-κB inhibition. Thus, it is possible to hypothesise a 'scenario' in which the cross talk between these two transcription factors, operated through the regulation of cyclooxygenase-2 gene expression (that may produce pro-inflammatory prostaglandin E_2 and anti-inflammatory prostaglandin J_2), modulates the inflammatory process.

In conclusion, our data wedge in another dowel in the complex mosaic of the molecular mechanisms underlying the onset and the remission of the inflammatory reaction and may represent the basis for understanding the protective effect of increase in body temperature during infection and its role in the resolution of inflammation.

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