



HSP70 increases extracellular matrix production by human vascular smooth muscle through TGF- β 1 up-regulation

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ABSTRACT

The circulating levels of heat shock proteins (HSP) are increased in cardiovascular diseases; however, the implication of this for the fibrotic process typical of such diseases remains unclear. HSP70 can interact with the vascular smooth muscle cells (SMC), the major producer of extracellular matrix (ECM) proteins, through the Toll-like receptors 4 (TLR4). The transforming growth factor type- β 1 (TGF- β 1) is a well known vascular pro-fibrotic cytokine that is regulated in part by AP-1-dependent transcriptional mechanisms. We hypothesized that extracellular HSP70 could interact with SMCs, inducing TGF- β 1 synthesis and subsequent changes in the vascular ECM. We demonstrate that extracellular HSP70 binds to human aorta SMC TLR4, which up-regulates the AP-1-dependent transcriptional activity of the TGF- β 1 promoter. This is achieved through the mitogen activated protein kinases JNK and ERK, as demonstrated by the use of specific blockers and the knockdown of TLR4 with specific small interfering RNAs. The TGF- β 1 upregulation increase the expression of the ECM proteins type I collagen and fibronectin. This novel observation may elucidate the mechanisms by which HSP70 contributes in the inflammation and fibrosis present in atherosclerosis and other fibrosis-related diseases.

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1. Introduction

Atherosclerosis is characterized by severe structural changes in vascular walls. The increased amount of abnormal extracellular matrix (ECM) proteins, much of which is produced by vascular smooth muscle cells (SMCs), is a common consequence of cardiovascular disease (Doran et al., 2008). Whereas most of the ECM proteins within healthy arteries are type I and type III fibrillar collagen, atherosclerotic lesions tend to contain mostly proteoglycans with scattered type I collagen fibrils and fibronectin (Ross, 1999). A developing body of evidence suggests that the

presence of circulating heat shock proteins (HSPs) is associated with a molecular cascade that results in the ECM pathological changes.

HSPs are a family of ubiquitous intracellular chaperones that are grouped and named into various families according to their molecular weight. The primary function of HSPs is to repair denatured proteins through folding/unfolding steps and thus achieve correct functional configuration (Young et al., 2004). However, the synthesis of HSPs is increased in response to many environmental stresses (Young, 2010).

These proteins are normally intracellular, but extracellular, soluble form has also been found in serum along with specific anti HSP antibodies (Asea, 2007). While the pathological significance of these circulating HSPs has not been adequately described, it is accepted that plasma HSPs levels reflect the accumulation of cellular damage. Circulating HSP60 and HSP70 are elevated in cardiovascular disease, and it is overexpressed in several cell types including SMCs (Kanwar et al., 2001). Additionally, there is a correlation between HSP70 plasma concentrations and the severity of

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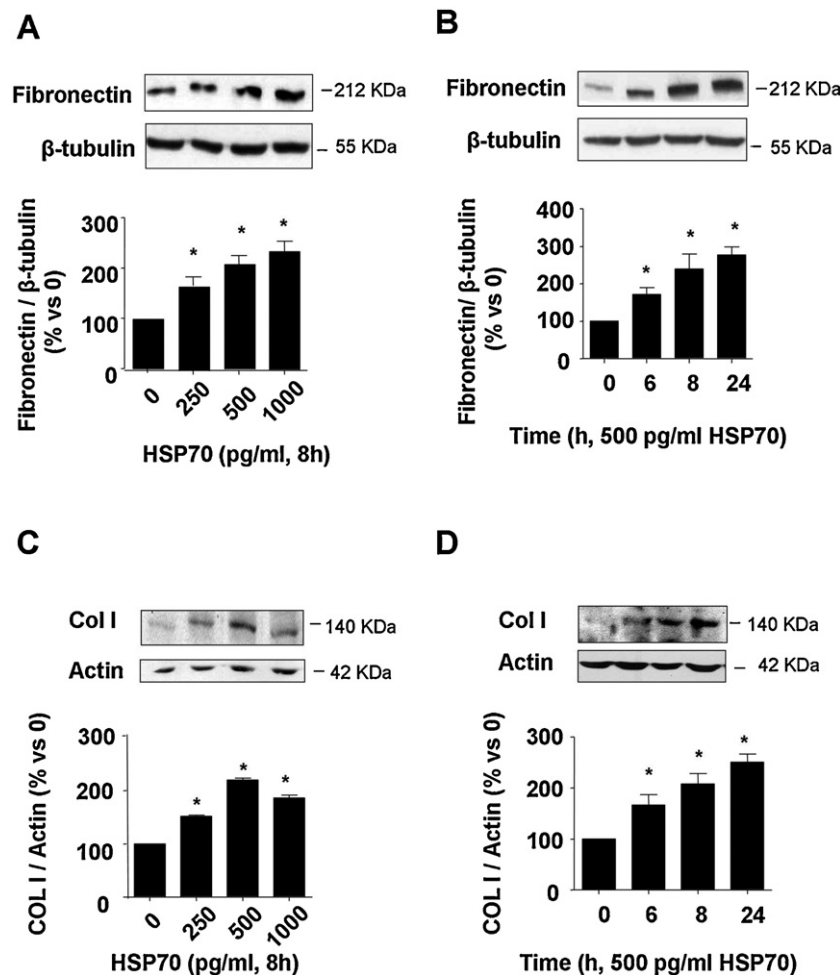


Fig. 1. HSP70 upregulates the expression of extracellular matrix proteins. Representative blots for fibronectin (A and B) or type I collagen (Col I, C and D) protein levels from SMC treated with HSP70 at different concentrations (A and C) and times (B and D). Bars represent the densitometric analysis from 6 independent experiments, normalized against endogenous control values from actin or β -tubulin content and expressed as mean% $\pm$ SEM. * $p < 0.05$ vs control (0).

atherosclerosis (Chan et al., 1999; Dulin et al., 2010; Krepuska et al., 2011; Pockley et al., 2003).

HSP70 may interact with membrane bound Toll-like receptors (TLR), inducing an increase of pro-inflammatory cytokine synthesis. The best-characterized TLRs involved in this regulation are TLR2 and TLR4 (Asea et al., 2002). TLR4 is present in SMCs (Yang et al., 2005), but the mechanisms by which it mediates extracellular HSP70-dependent vascular pro-inflammatory cytokine synthesis are poorly established. Some studies implicate the mitogen-activated protein kinases (MAPK) p38, ERK and/or JNK signalling pathways (Gabai et al., 1997; Jones et al., 2001; Song et al., 2001). Alternatively, phosphatidylinositol 3-kinase (PI3K), which phosphorylates serine/threonine kinase protein kinase B (PKB, also known as AKT) has been also proposed (Cai et al., 2010).

Transforming growth factor type- β 1 (TGF- β 1) is a locally expressed pro-fibrotic cytokine that regulates growth, differentiation, migration and proliferation of SMC as well as ECM protein synthesis (Ruiz-Ortega et al., 2007). TGF- β 1 plays an integral role in promoting intimal thickening by stimulating abnormal ECM protein expression such as fibronectin and type I collagen and SMC proliferation and migration, leading to eventual luminal narrowing (Bobik, 2006). Different factors involved in the development and maintenance of cardiovascular damage up-regulate SMC TGF- β 1 synthesis and post-translational activation (Annes et al., 2003; Doran et al., 2008). Interestingly, HSP70 has been established as a regulator in the TGF- β 1-induced epithelial-mesenchymal

transition (Li et al., 2011). The transcriptional regulation of TGF- β 1 gene includes activator protein-1 (AP-1) (Kim et al., 1990) and NF- κ B, possibly through activation of TLR4 (Lee et al., 2006). Furthermore, TLR activation regulates AP-1 and NF- κ B promoter activity through MAPK (Edfeldt et al., 2002; Faure et al., 2000; Ji et al., 2009; Jones et al., 2001).

Considering all these interactions, we hypothesize that in pathological conditions, circulating HSPs interact with SMCs to induce pro-fibrotic TGF- β 1 synthesis. This interaction, in turn, results in subsequent changes in the vascular structure. In the present work, we demonstrated for the first time that extracellular HSP70 regulates the pro-fibrotic response of human aorta SMCs through increased TGF- β 1 expression. HSP70 binds to TLR4 activating ERK and JNK. These MAPKs are implicated in the transcriptional activation of the TGF- β 1 promoter by increasing AP-1, but not NF- κ B transcriptional response. The final consequence is a TGF- β 1-dependent abnormal increased ECM proteins synthesis by the SMCs.

2. Materials and methods

2.1. Reagents

Dulbecco's modified Eagle's medium and Foetal bovine serum were from Lonza (Basel, Switzerland). Antibiotics, goat anti-rabbit antibody, lipofectamine, opti-MEM were from Invitrogen

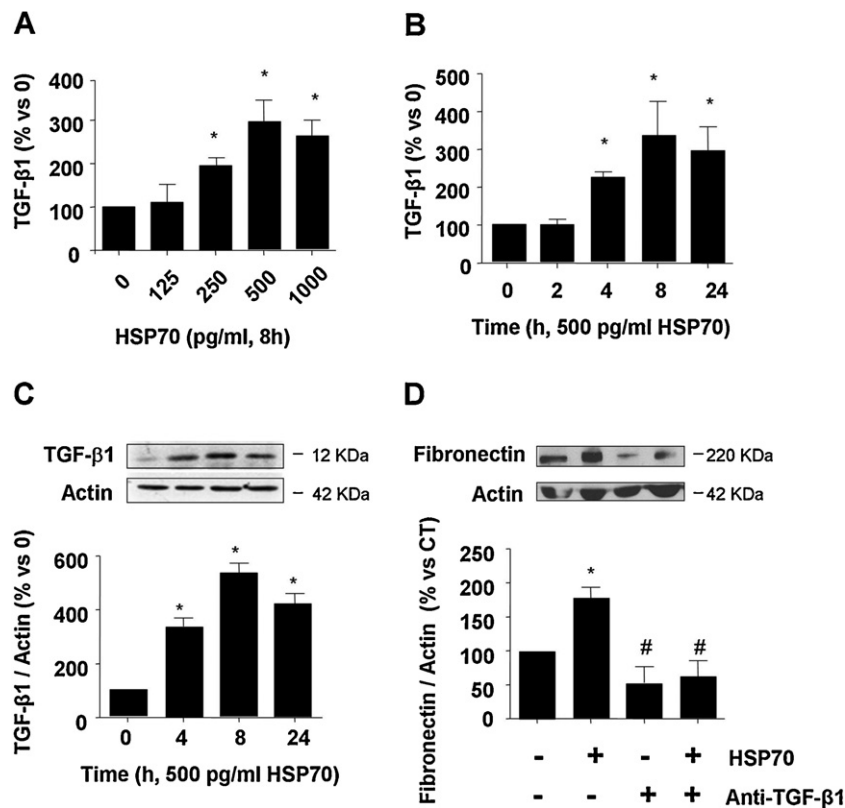


Fig. 2. HSP70 upregulates the expression of fibronectin through TGF-β1. Extracellular TGF-β1 levels were determined by ELISA in the supernatant of SMC treated with HSP70 at different concentrations (A) and times (B). (C) Representative TGF-β1 immunoblot from SMC treated with 500 pg/ml HSP70 during different times. (D) Representative fibronectin immunoblot from SMC pre-treated with a specific TGF-β1 blocker antibody (anti-TGF-β1, 30 μg/ml 1 h) and treated with 500 pg/ml HSP70 during 8 h. Bars represent the analysis from 6 or more independent experiments, normalized against protein content or actin densitometry as endogenous control values in ELISA or immunoblot, respectively, and expressed as mean% ± SEM. **p* < 0.05% vs control (0). #*p* < 0.05% vs HSP70.

(Life Technologies, Ltd., Paisley, UK). Antibodies against p38, phospho-p38, AKT, phospho-threonin 308 AKT, extracellular signal-regulated protein kinases 1/2 (ERK), phospho-ERK, c-Fos, c-Jun and phospho-c-Jun were from Cell Signaling Technology (Boston, MA, USA). High Capacity cDNA Reverse Transcription Kit and TaqMan gene expression assays were purchased from Applied Biosystems (Life Technologies, Ltd., Paisley, UK). Human TGF-β1 antibody, TLR4 antibodies and Signal-Silencer TLR4 siRNA were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Silencer negative control siRNA was from Ambion (Life Technologies, Ltd., Paisley, UK). PI3K inhibitor, HSP70 antibody, PD98059 were from Enzo Life Science, Inc. (Farmingdale, NY, USA). Anti-rabbit horseradish peroxidase antibody, SP600125 and SB203580 were from Chemicon (Millipore Corporation, Billerica, MA, USA). Dual-Luciferase Reporter Assay System and ELISA kit and renilla luciferase plasmid were from Promega (Madison, WI, USA). BCA protein assay reagent was from Pierce (Rockford, IL, USA). Nylon membrane, non-immune IgG and protein G-Sepharose were from Amersham Biosciences (GE Healthcare Biosciences, Pittsburgh, PA USA). Anti-TGFβ blocking antibody was from R&D systems (Minneapolis, MN, USA). PVDF is from Perkin Elmer (Waltham, MA, USA). Enhanced chemiluminescent reagent (ECL), TGF-β1 ELISA kit, X-ray films and Lightshift chemiluminescent EMSA kit were from Thermo Fisher Scientific (Waltham, MA, USA). Human recombinant HSP70 and other antibodies and reagents were from Sigma-Aldrich Chemical Company (St. Louis, MO, USA).

2.2. Cell culture

Human aorta smooth muscle cells (SMCs) were obtained by enzymatic dissociation from the aortas of five organ donors,

according to Spanish legal dispositions and the Ethics Committee of the Hospital Universitario de Getafe, Spain, as previously described and kindly provided by Dr. Sanchez-Ferrer (Peiró et al., 2003). Cells were cultured in DMEM supplemented with 10% FBS and antibiotics. Confluent, 24 h serum-deprived SMC from 3 to 10 passages were used.

2.3. Immunoblot assay

Immediately after treatments, cells were lysed and sonicated. Proteins samples were run onto SDS-PAGE gels and transferred to PVDF membranes. The membranes were blocked and incubated with the corresponding primary antibodies and secondary antibodies afterwards. Immunoblots were detected by enhanced chemiluminescence and densitometrically analyzed by using ImageJ software.

2.4. Immunoprecipitation assay

This technique has been described previously (Ortega-Velazquez et al., 2004). Briefly, extracted proteins from SMC were pre-adsorbed with non-immune IgG and incubated with TLR4 antibody. The antigen-antibody complexes were precipitated with G-Sepharose. Proteins from pelleted beads were run onto SDS-PAGE gels, transferred to PVDF membranes, and blotted against HSP70, or TLR4 as loading control, as described above.

2.5. Elisa assay

The supernatant from cultured SMC was incubated with 1 N HCl to activate latent TGF-β1, neutralized and analyzed with a

commercial TGF- β 1 ELISA kit according to the manufacturer's recommendations.

2.6. Quantitative RT-PCR

RNA extracted from SMC was reverse transcribed with High Capacity cDNA Reverse Transcription Kit, and TGF- β 1 and GAPDH complementary DNA were amplified by quantitative PCR by using TaqMan gene expression assay on 96-well plates using the 7500 Fast Real-Time PCR System and analyzed with 7500 Fast sequence detection software v1.3.1 (Applied Biosystems, California USA).

2.7. Transient cDNA and siRNA transfection

SMC were lipofectamine-transfected with the specific siRNA against TLR4 or unspecific siRNAs or reporter plasmids, and processed for immunoblotting or luciferase activity measurement with a commercial Dual-Luciferase Reporter Assay System according to the manufacturer's recommendations. The reporter plasmids used were AP-1-sensitive (3TPLux) or NF- κ B-sensitive (3XNRE) reporter plasmids (Saura et al., 2005) and the complete promoter region of the human TGF- β 1 gene linked to the luciferase reporter gene and deletion mutants pHG5 and pHG6, all kindly provided by Dr. Kim (Kim et al., 1989). All the luciferase reporter plasmids were co-transfected with commercial renilla luciferase reporter plasmid as control.

2.8. Electrophoretic mobility shift assay (EMSA)

To detect DNA-transcription factor interactions, non-isotopic EMSA kit was performed following the manufacturer instructions. Briefly, 5 μ g nuclear extracts from SMC treated or not with 500 pg/ml HSP70 at different times were probed with a 3' biotin end-labelled oligonucleotide (5'-CTT TCC CAG CCT GAC TCT CCT TCC GTT C-3') which contains the putative AP-1 binding sequence at position -418 (Kim et al., 1989) into the human TGF- β 1 promoter (Ruiz-Torres et al., 2007).

DNA-protein complexes were subjected to gel electrophoresis on a native polyacrylamide gel and transferred to a positively charged nylon membrane. The biotin end-labelled DNA was detected using streptavidin-horseradish peroxidase conjugate with the chemiluminescent substrate provided by the kit. To determine binding specificity, competition experiments were performed by using nuclear extracts from control SMC, where 200-fold molar excess of non-labelled competitor DNAs were co-incubated with the Biotin end-labelled DNA for AP-1. The competition DNAs sequences used were the same AP-1 oligonucleotide or a NF- κ B-binding sequence (5'-GGC TGG AGG GAT GAT TTT TCC TCC TTT CA-3'), both non-biotin-labelled.

2.9. Statistical analysis

Results are expressed as the mean $\pm$ SEM. Statistical significance was tested at 95% ($p < 0.05$) confidence level using the Friedman test.

3. Results

3.1. Extracellular HSP70 increases ECM proteins levels by a TGF- β 1-dependent mechanism

Extracellular HSP70 treatments increased fibronectin (Fig. 1A and B) and collagen I (Fig. 1C and D) synthesis by SMC in a concentration- and time-dependent manner. Significant increases occurred as early as 6 h after treatment with a minimum of 250 pg/ml of HSP70. In addition, the presence of extracellular

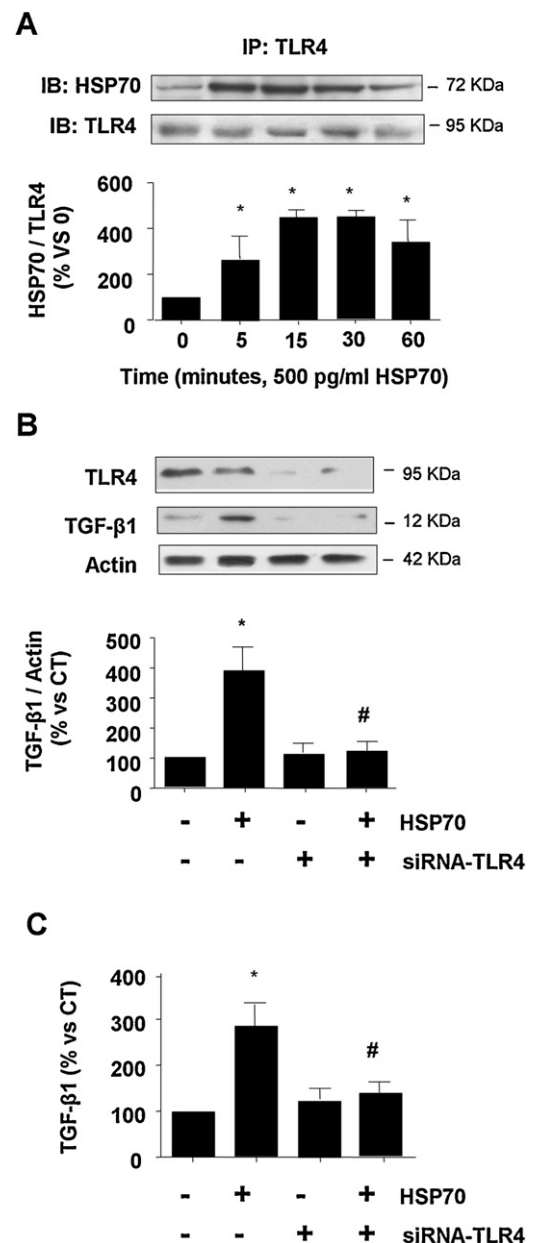


Fig. 3. The upregulation of TGF- β 1 by HSP70 is mediated by TLR4. (A) SMC treated with 500 pg/ml HSP70 for 5–60 min were immunoprecipitated with TLR4 antibody (IP: TLR4) and immunoblotted (IB) against HSP70 or TLR4 to normalize IP HSP70 values. (B) TGF- β 1 immunoblot or (C) TGF- β 1 supernatant ELISA from SMC transiently transfected with siRNAs against TLR4 (siRNA-TLR4) or with scrambled siRNA negative control and treated with or without HSP70 (500 pg/ml, 8 h). TLR4 immunoblot shows the TLR4 depletion in siRNA transfected cells. Bars represent the analysis from 6 or more independent experiments, normalized against protein content or actin densitometry as endogenous control values in ELISA or immunoblot, respectively, and expressed as mean $\pm$ SEM. * $p < 0.05$ vs control (CT). # $p < 0.05$ vs HSP70.

HSP70 increased the total immunoreactive TGF- β 1 produced by SMCs in a concentration- and time-dependent manner as detected in supernatants by ELISA (Fig. 2A and B). The minimal concentration of HSP70 that elicited a significant increase in TGF- β 1 synthesis as well as the minimal time of incubation both correlated with the up-regulation of ECM proteins. Finally, we confirmed the increased TGF- β 1 protein synthesis by Western blot (Fig. 2C).

We verified the mediating role of TGF- β 1 in this HSP70-dependent fibronectin up-regulation by blocking the signalling pathway with 30 μ g/ml of anti-TGF- β 1 antibody (Tsang et al., 1995). The antibody was applied 1 h prior to treatment with HSP70,

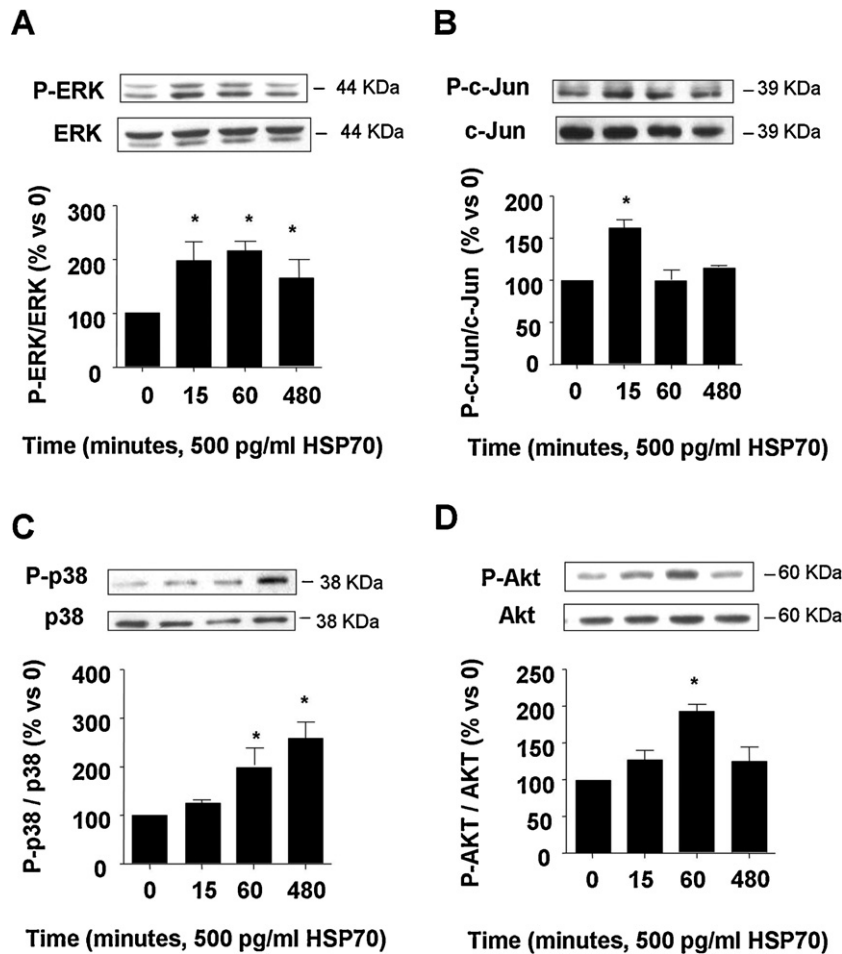


Fig. 4. PI3K and the MAPKs ERK, JNK and p38 phosphorylating activities in HSP70-treated SMC. Representative immunoblots against phosphorylated ERK (P-ERK, A), c-Jun (P-c-Jun, substrate of JNK, B), p38 (P-p38, C) or AKT in the threonine 308 residue (P-AKT, substrate of PI3K, D) from SMC treated with HSP70 (500 pg/ml) during different times. The corresponding total ERK, c-Jun, p38 or AKT protein levels were blotted as endogenous controls. Bars represent the densitometric analysis from 6 independent experiments, normalized against their respective endogenous control values and expressed as mean $\pm$ SEM. * $p < 0.05$ vs control (0).

and the increased fibronectin synthesis was inhibited (Fig. 2D). These results demonstrate that TGF- β 1 is the mediator in the HSP70-induced up-regulation of some ECM proteins.

3.2. HSP70-mediated TGF- β 1 up-regulation is mediated by TLR4

Because extracellular HSP70 binds to TLR4 (Asea et al., 2002), which is present in SMC (Yang et al., 2005), we analyzed the possibility that this receptor could mediate in the TGF- β 1 changes induced by HSP70. Thus, HSP70-treated SMC were lysed, the proteins were immunoprecipitated with a specific TLR4 antibody, and the separated proteins were blotted against HSP70 or TLR4 with specific antibodies. HSP70 was bound to TLR4 quickly and remain sustained for at least 1 h (Fig. 3A). To confirm the importance of TLR4 mediating the HSP70-dependent TGF- β 1 up-regulation, SMC were transfected with TLR4 small interfering RNAs (siRNAs) prior to the HSP70 treatment. The siRNA treatment knocked-down the expression of TLR4, and HSP70 treatment in TLR4-depleted cells did not up-regulate either the intracellular (Fig. 3B) or secreted (Fig. 3C) levels of TGF- β 1.

3.3. Intracellular kinases implicated in the HSP70-induced TGF- β 1 up-regulation

SMCs were treated with HSP70 for short periods of time, and the activation of the potential kinases implicated in the process of

TGF- β 1 up-regulation, such as MAPK or PI3K/AKT, were analyzed by Western blot. Within 15 min after treatment, the substrate for JNK MAPK, c-Jun, and ERK were both rapidly phosphorylated after HSP70 treatment (Fig. 4A and B). In contrast, the phosphorylation of MAPK p38 was significant only after 1 h of HSP70 incubation (Fig. 4C). After 1 h of HSP70 treatment, AKT was also phosphorylated at threonine 308, the canonical phosphorylation site for PI3K (Duronio, 2008) (Fig. 4D). Thus, several kinases were activated at different times after HSP70 treatment.

We determined if these kinases were implicated in the specific HSP70-dependent TGF- β 1 up-regulation by pre-treating the cells for 8 h with pharmacological kinases inhibitors. After HSP70 treatment, we analyzed by ELISA the levels of TGF- β 1 in the supernatants. Inhibition of ERK phosphorylation by 50 μ M of the specific MAPK kinase (MEK) inhibitor PD98059 (Alessi et al., 1995) significantly reduced the HSP70-dependent TGF- β 1 up-regulation (Fig. 5A). Similarly, inhibition c-Jun phosphorylation by 10 μ M of the specific JNK inhibitor SP600125 (Moon et al., 2005) had the same effect (Fig. 5B). In contrast, inhibition of p38 by 25 μ M of the SB203580 (Thiel et al., 2006) had no effect on HSP70-induced TGF- β 1 up-regulation (Fig. 5C). Similarly, inhibition of PI3K by 30 μ M of a phosphatidylinositol analogue (PI3K INH), which in turn would inhibit threonine 308 phosphorylation of AKT (Hu et al., 2000), had no effect on HSP70-induced TGF- β 1 up-regulation (Fig. 5D).

Because fibronectin up-regulation mediated by HSP70 is a cause of the TGF- β 1 induction, we strengthened the link between

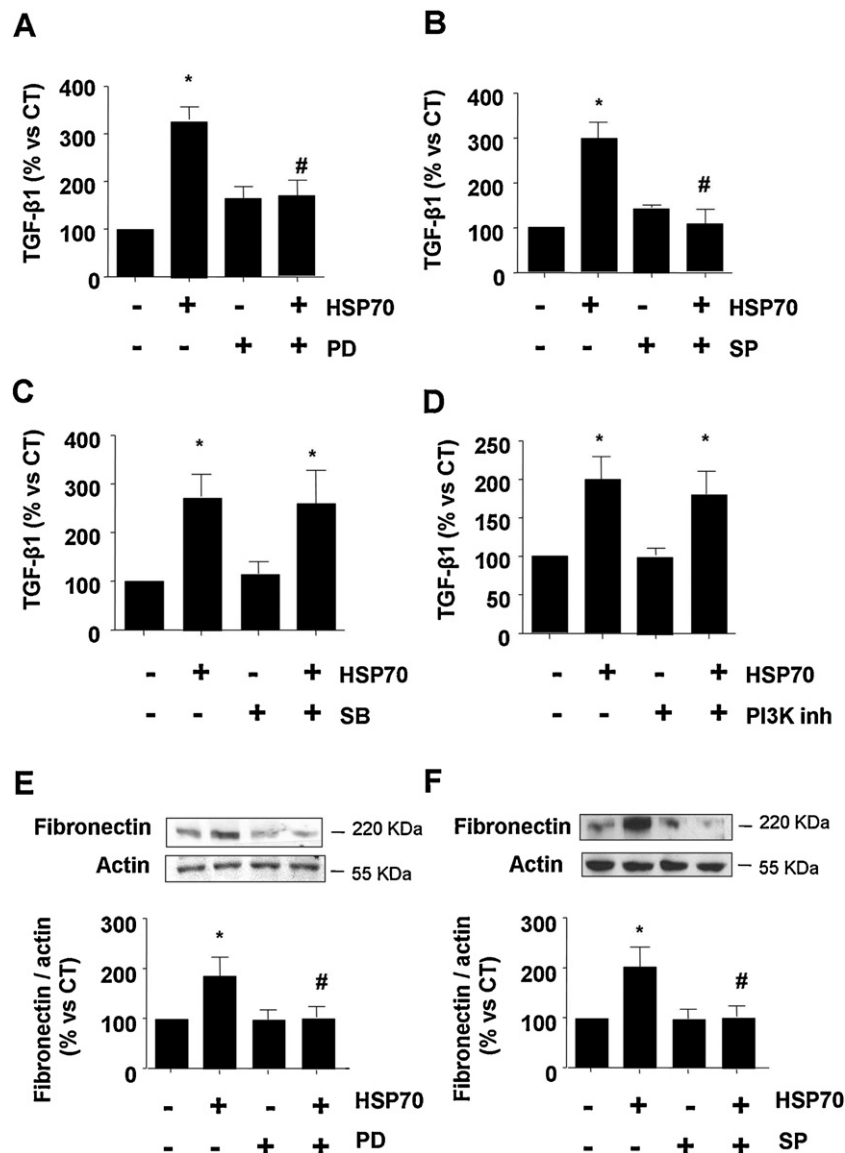


Fig. 5. PI3K and the MAPKs ERK, JNK and p38 activities in the HSP70-mediated TGF- β 1 upregulation. Extracellular TGF- β 1 levels were determined by ELISA in the supernatant from SMC co-treated with or without HSP70 (500 pg/ml, 8 h) and the specific ERK inhibitor PD98059 (PD, 50 μ M, A), the specific JNK inhibitor SP600125 (SP, 10 μ M, B), the specific p38 inhibitor SB203580 (SB, 25 μ M, C) or PI3K inhibitor (PI3K Inh, 30 μ M, D). Fibronectin levels were determined by immunoblot in SMC co-treated with HSP70 (500 pg/ml, 8 h) and (E) PD98059 (PD, 50 μ M) or (F) SP600125 (SP, 10 μ M). Actin protein levels were blotted as endogenous controls. Bars represent the results from at least 6 independent experiments, normalized against the total protein in ELISA or against the actin protein levels in immunoblots. All the values are expressed as mean $\pm$ SEM. * p < 0.05% vs control. # p < 0.05% vs HSP70.

them by determining fibronectin expression levels after HSP70 treatment in the presence of the MEK inhibitor PD98059 and the JNK inhibitor SP600125. Both reagents significantly inhibited the HSP70-mediated fibronectin up-regulation (Fig. 5E and F). Because HSP70 mediates this effect through binding to TLR4, we confirmed the link between TLR4 and ERK and JNK by determining the activity of these kinases in cells where TLR4 was knock-down with specific siRNAs. The activity of both MAPKs was reduced in siRNA-TLR4 transfected cells after HSP70 treatment (Fig. 6A and B). These results show that HSP70 activates a pool of kinases, but not all of them mediate the HSP70-dependent TGF- β 1 up-regulation. Only the inhibition of ERK and JNK MAPKs reversed that up-regulation, while neither p38 MAPK nor PI3K inhibition did so. The binding of HSP70 to TLR4 is a necessary event upstream from MAPK activation. Inhibition of these kinases results in the inhibition of the HSP70-dependent fibronectin production.

3.4. HSP70-dependent TGF- β 1 upregulation is transcriptionally regulated by AP-1

The next step in the analysis of the mechanisms mediating the HSP70-dependent TGF- β 1 up-regulation was to measure the TGF- β 1 mRNA levels under HSP70 treatment and to analyze the transcriptional mechanisms involved. As expected, the mRNA levels of TGF- β 1 were increased as soon as 1 h after HSP70 treatment (Fig. 7A). Co-treatment with the transcriptional inhibitor 5,6-dichloro-1- β -D-ribofuranosylbenzimidazole (DRB) blocked this up-regulation (Fig. 7B).

Furthermore, we wanted to study the specific nuclear transcription factors that may be regulating the transcriptional activity of TGF- β 1 promoter, which contain at least two AP-1 binding sequences (Lee et al., 2006) that are regulated by MAPK-dependent mechanisms (Naito et al., 2004; Ortega-Velazquez et al., 2004; Ruiz-Ortega et al., 2007).

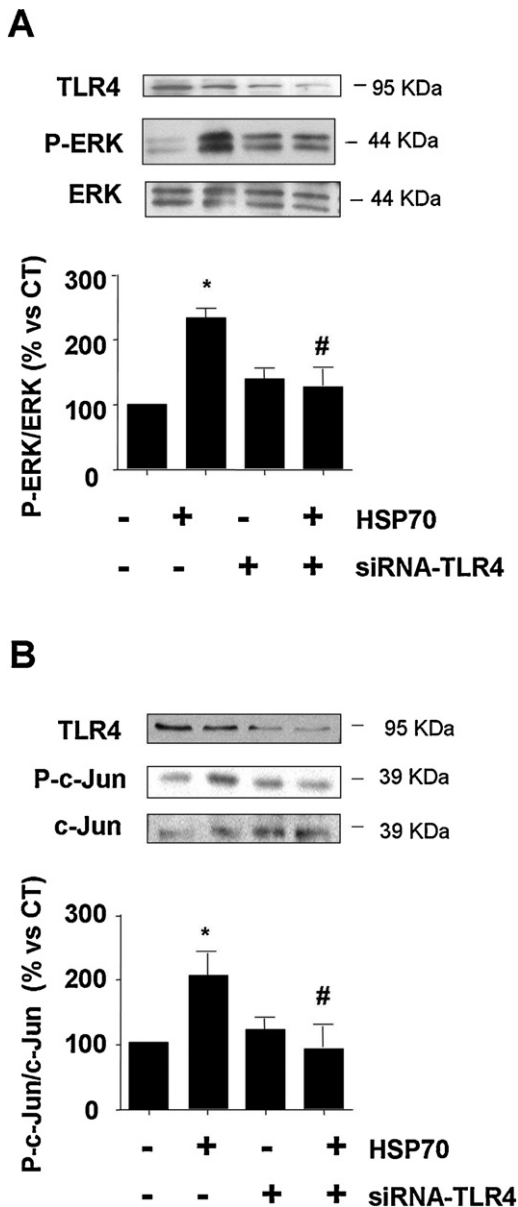


Fig. 6. The MAPKs ERK and JNK activities in HSP70-mediated effect is regulated by TLR4. Representative immunoblots against phosphorylated ERK (P-ERK, A) or c-Jun (P-c-Jun, B) from SMC transiently transfected with siRNAs against TLR4 (siRNA-TLR4) or with scrambled siRNA negative control and treated with or without HSP70 (500 pg/ml, 15 min). TLR4 immunoblot shows the TLR4 depletion in siRNA transfected cells. The corresponding total ERK or total c-Jun protein levels were blotted as endogenous controls. Bars represent the densitometric analysis from 6 or more independent experiments, normalized against their respective endogenous control values and expressed as mean% $\pm$ SEM. * $p < 0.05$ vs control. # $p < 0.05$ vs HSP70.

The ability of HSP70 stimulating AP-1 activity was confirmed since HSP70 increased in a concentration dependent manner the activity of the 3TP-Lux plasmid (González-Ramos et al., 2012), a reporter plasmid in which luciferase activity is controlled by three AP-1 binding sites (Fig. 7C). After 24 h, luciferase activity induced by HSP70 was equivalent to that induced by 3 nM of phorbol-12-myristate-13-acetate (PMA), a positive control for the AP-1 transcriptional activity.

In contrast, HSP70 did not modify the luciferase activity from 3XNRE, a NF- κ B-controlled reporter plasmid (Fig. 7D), which did response to bacterial lipopolysaccharide (LPS, 100 ng/ml, 24 h) used as a positive control (Edfeldt et al., 2002; Faure et al., 2000).

Next, we undertook a more detailed analysis of the AP-1-dependent transcriptional activity of the TGF- β 1 promoter. Luciferase activity was measured after the transient transfection of SMCs with a plasmid in which the luciferase gene was attached to complete, double TGF- β 1 promoter sequences, with two described AP-1-binding sites. A second construct, phTG5, included a deletion of the TGF- β 1 first promoter, but retained a single AP-1 binding sequence at position -418 (Kim et al., 1989). The third construct, phTG6, included another deletion of the TGF- β 1 first promoter that contained no AP-1 binding site.

HSP70 up-regulated the complete TGF- β 1 promoter activity as well as the first promoter-mutated construction, phTG5, both containing at least one AP-1 site. However, neither HSP70 nor PMA up-regulated the activity of SMCs transfected with phTG6 plasmid, in which there was no consensus sequence for AP-1 binding (Fig. 8A). We confirmed the binding of HSP70-activated AP-1 to the described binding site at the position -418 in TGF- β 1 first promoter by specific EMSA analysis of the binding sequence (Fig. 8B) (Ruiz-Torres et al., 2007). Increased AP-1 binding was observed to the labelled TGF- β 1 sequence as early as 15 min after HSP70 treatment. The non-labelled oligonucleotides competition experiments demonstrated the AP-1-oligonucleotide binding specificity (Fig. 8C).

4. Discussion

While HSPs have long been considered as a classical intracellular mechanism for cell protection (Kanwar et al., 2001; Young et al., 2004), more recently several authors have shown that circulating HSPs elicit specific immune reactions that lead to the formation of antibodies (Asea et al., 2002; Multhoff and Hightower, 2011). For HSP70, most studies have proposed that the circulating protein and associated antibodies are markers for cardiovascular disease-related cellular damage, such as atherosclerotic lesions (Chan et al., 1999; Dulin et al., 2010; Krepuska et al., 2011; Pockley et al., 2003). However, the increased levels of circulating HSPs are not merely a marker, but an active mediator in the inflammatory process present in the cardiovascular damage. Rather, through interactions with membrane-bound receptors such as TLR4, they actively initiate the synthesis of pro-inflammatory cytokine (Edfeldt et al., 2002; Faure et al., 2000; Ji et al., 2009; Yang et al., 2005).

In the present work, we tested the hypothesis that circulating HSP70 acts as a pro-fibrotic regulator of the ECM proteins synthesis, particularly fibronectin and type I collagen, through changes in the cytokine TGF- β 1 production. We showed by different techniques that extracellular HSP70 increased the expression of TGF- β 1 in SMCs. When SMCs were incubated with HSP70, we observed by immunoblotting the increased production of fibronectin and type I collagen. Furthermore, we inhibited the increased fibronectin production by blocking the HSP70-dependent TGF- β 1 up-regulation with anti-TGF- β 1 blocking antibody.

In the analysis of these results, we pose a very relevant question concerning the pathophysiological meaning of the extracellular HSP70 concentrations that we used in our in vitro experiments. The mediator concentration in the pericellular environment is not necessarily the same as the circulating concentration, but a pathophysiological phenomenon may be better explained when both concentrations are approached. In our in vitro experiments, 250 pg/ml of HSP70 induced a significant cellular response, and maximum stimulation was achieved at 500–1000 pg/ml. This range of concentrations has been described in plasma from human in several studies. Some authors consider the high circulating HSP70 concentrations as unfavourable (Nakhjavani et al., 2012) while others suggest that the high levels could be protective (Dulin et al., 2010; Pockley et al., 2009). However, to our knowledge, no work has

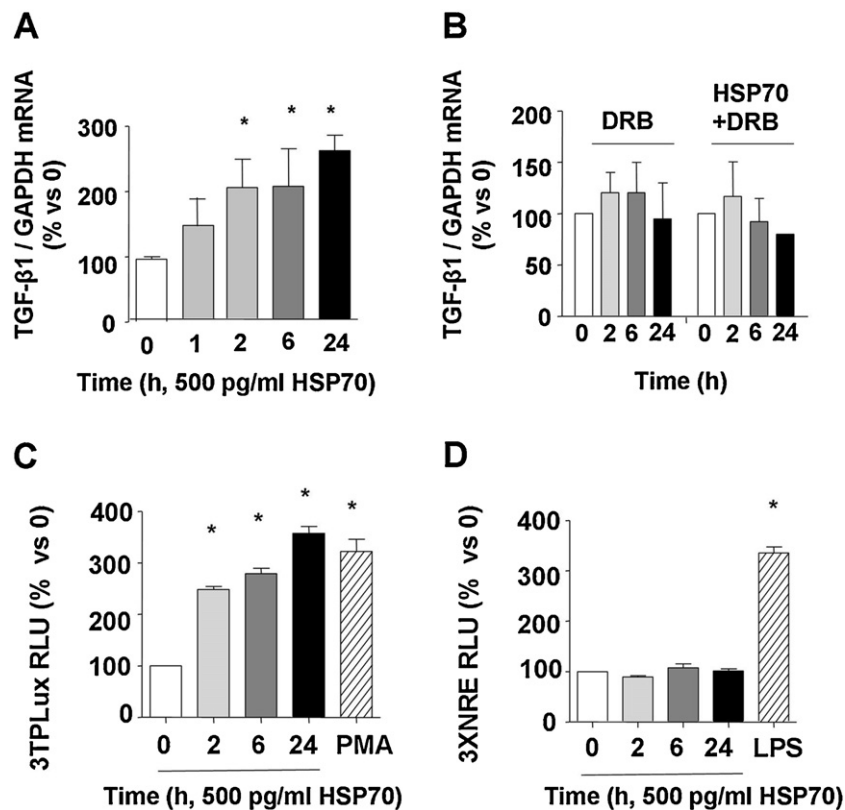


Fig. 7. AP-1 but not NF- κ B transcriptional activity in HSP70-treated SMC. (A) mRNA levels of TGF- β 1 were determined by RT-quantitative-PCR from SMC treated with 500 pg/ml HSP70 at different times or (B) co-treated with the transcription inhibitor DRB (10 μ M). Relative fold change values were normalized against GAPDH as endogenous control. (C) SMC were co-transfected with plasmids containing renilla and AP-1-activity reporter plasmid (3TPLux) or (D) NF- κ B-activity reporter plasmid (3XNRE). Transfected cells were treated with 500 pg/ml HSP70 during different times. Relative luciferase vs renilla activities were determined as relative luciferase units (RLU). 24 h 3 nM PMA- or 100 ng/ml LPS-treated transfected SMC were used as positive controls for AP-1 or NF- κ B transcriptional activity, respectively. Bars in the figures represent the results from more than 6 independent experiments, expressed as mean $\pm$ SEM. * p < 0.05% vs control (0). # p < 0.05% vs TGF- β 1.

identified a pro-fibrotic mechanism for the circulating HSP70 in the vascular SMCs. According to our results, we suggest that increased HSP70 circulating levels may induce TGF- β 1 overexpression and the subsequent increase in ECM protein synthesis and fibrosis. Interestingly enough, our observations confirm a recently established link between HSP70 and TGF- β 1-dependent cellular effects as reported by Li et al. (2011). They found that HSP70 regulates the TGF- β 1-induced epithelial–mesenchymal transition.

We explored the mechanisms involved in the extracellular HSP70-dependent TGF- β 1 up-regulation, such as the specific membrane receptor that binds to HSP70, the signalling cascade and the transcription factors responsible for the changes in the TGF- β 1 levels. We based our studies in previously published evidences concerning the relevance of the SMC receptor for microbial pathogen-associated proteins TLR4 (Edfeldt et al., 2002; Faure et al., 2000; Ji et al., 2009; Yang et al., 2005), different kinases (Cai et al., 2010; Gabai et al., 1997; Jones et al., 2001; Song et al., 2001) and transcription factors (Edfeldt et al., 2002; Faure et al., 2000; Ji et al., 2009; Jones et al., 2001; Lee et al., 2006; Naito et al., 2004).

To determine if the initial event in extracellular HSP70-induced ECM protein synthesis was binding of HSP70 to SMCs TLR4, we knocked-down TLR4 employing specific siRNAs. The loss of TLR4 resulted in the inability of HSP70 to increase the synthesis of the key cytokine TGF- β 1. To elucidate which kinases mediated the HSP70-dependent TGF- β 1 up-regulation, we used specific kinases inhibitors. We found that only ERK and JNK mediated the HSP70-induced TGF- β 1 up-regulation, even though other kinases that have being also related with the signalling of HSP70 or TGF- β 1 induction, such as p38 (Cai et al., 2010) and PI3K/AKT (Gille et al., 1992), were

also active after HSP70 treatment, but not mediated the HSP70-induced TGF- β 1 up-regulation.

ERK and JNK are the kinases responsible of c-Fos and c-Jun phosphorylation, respectively (Gille et al., 1992). Both phosphorylated proteins form the transcriptional factor AP-1 that regulates TGF- β 1 by binding to specific sites as we and others have shown (Tsang et al., 1995; Iglesias-De La Cruz et al., 2001; Ortega-Velazquez et al., 2004; Rhyu et al., 2005; Ruiz-Torres et al., 2007; Saura et al., 2005; González-Ramos et al., 2012).

TLR4 downstream signalling depends on different molecules such as myeloid differentiation primary-response protein-88 (MyD88) or TIR domain-containing adaptor protein (TRIF) (Kawai and Akira, 2010) that ultimately lead to the activation of MAPKs (Newton and Dixit, 2012). In cultured human SMC, TLR4 activates AP-1 via MyD88 and the TNF receptor-associated factor 6 (TRAF6) among other mediators (Patel et al., 2006). However, further studies are necessary to elucidate the role of these mediators in the HSP70-dependent ERK and JNK activation.

We analyzed the HSP70-dependent AP-1 transcriptional activity by using different promoter plasmids. First, HSP70 stimulated phospholuminescence of the 3TPLux plasmid construct, which required activation by AP-1. This occurred without the activity of the transcriptional factor NF- κ B, although it is a well-defined TGF- β 1-transcriptional activator (Allen and Tresini, 2000) that is also activated by TLRs in SMCs (Edfeldt et al., 2002; Ji et al., 2009) and other cell types (Faure et al., 2000; Jones et al., 2001). Furthermore, we used TGF- β 1 promoter plasmids with serial deleted sequences. Both the complete promoter and phTG5 plasmid construct, with a deletion of the region between –1362 and –453, increased the

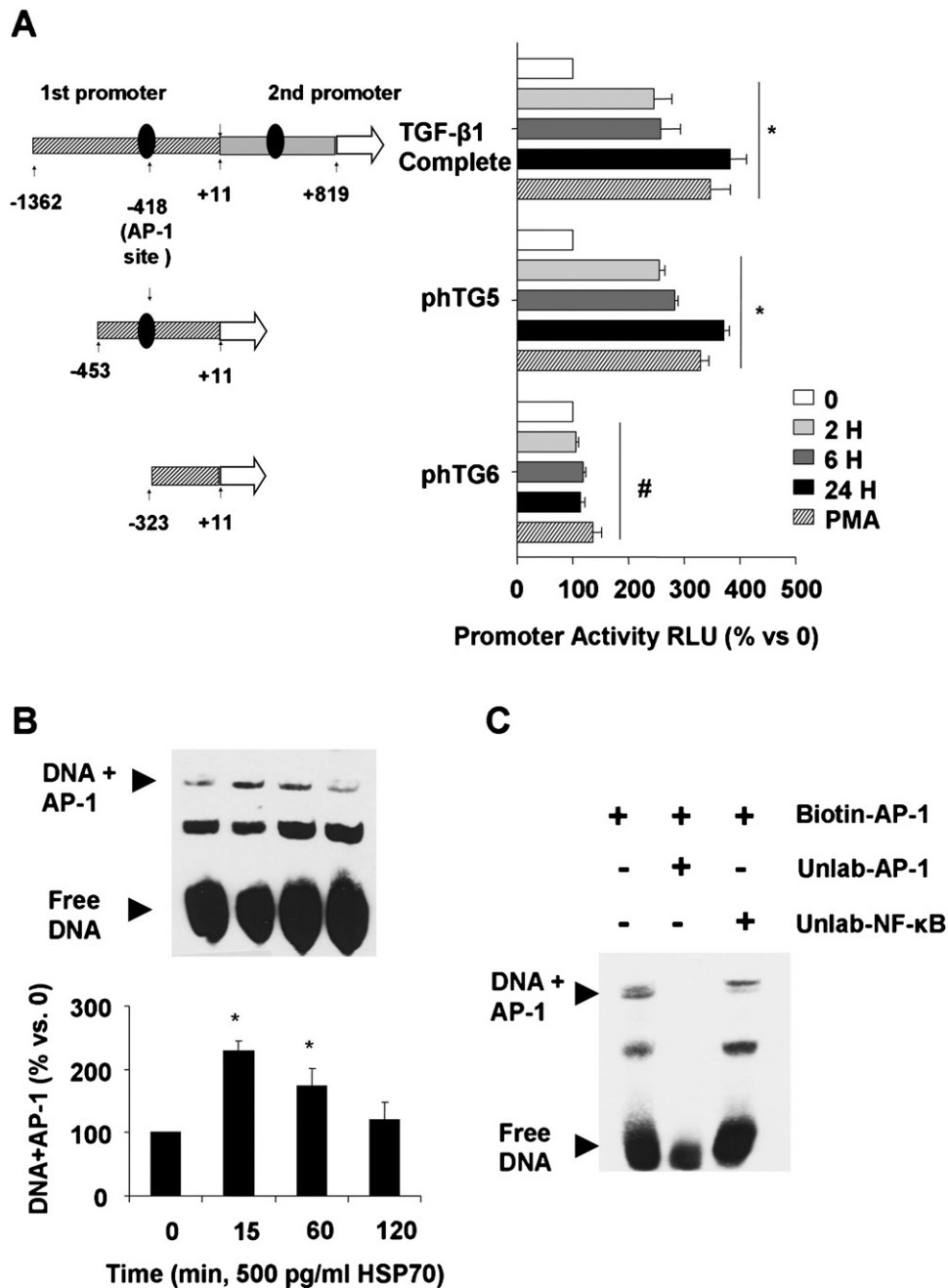


Fig. 8. HSP70-dependent TGF- β 1 transcriptional activity is mediated by AP-1 binding to -418 position located into the TGF- β 1 promoter. (A) SMC were co-transfected with plasmids containing renilla and the complete sequence of the TGF- β 1 promoters (TGF- β 1) or mutants (phTG5 or phTG6) with serial-deleted sequences of the human TGF- β 1 first promoter linked to the luciferase reporter gene. A representation of the 5'-flanking sequence of the complete and mutated human TGF- β 1 promoters-containing plasmids are shown; the two deletion mutants were truncated at the locations shown and AP-1 binding site of the first promoter is located at -418. The white arrow represents the linked luciferase reporter gene. Transfected cells were treated with 500 pg/ml HSP70 during different times. Relative luciferase vs renilla activities were determined as relative luciferase units (RLU). 24 h 3 nM PMA-treated transfected SMC were used as positive controls for AP-1 transcriptional activity. (B) Nuclear proteins were extracted from SMC treated with 500 pg/ml HSP70 at different times and EMSA was performed by incubating 5 μ g of the nuclear extracts with biotin end-labelled DNA sequence for AP-1 at -418 position of the human TGF- β 1 promoter. A representative EMSA and the densitometric analysis of the biotin labelled DNA bound to AP-1 bands are shown (DNA + AP-1). (C) Representative EMSA from 4 different competition experiments performed by using nuclear extracts from control SMC incubated with the biotin labelled DNA for AP-1 (biotin-AP-1), with or without an excess of unlabelled AP-1-DNA sequence (unlab-AP-1) or unlabelled NF- κ B (unlab-NF- κ B). Bars in the figures represent the results from 6 independent experiments, expressed as mean $\pm$ SEM. * p < 0.05% vs control (0). # p < 0.05% vs TGF- β 1.

transcription activity in response to HSP70. However, the phTG6 plasmid construct, with a further deletion including the fragment between -453 and -323 did not respond to HSP70. This deletion results in the loss of the AP-1 binding site at the position -418 in the first promoter region of TGF- β 1 (Kim et al., 1989). Our group has previously demonstrated the specific binding of AP-1 in this site of the human TGF- β 1 promoter after different stimuli (Ruiz-Torres

et al., 2007; González-Ramos et al., 2012). Here we also demonstrated by using EMSA analysis that HSP70 increased the binding of AP-1 in that region of the TGF- β 1 promoter in human SMC.

All together, these results may clarify how HSP70 stimulates TGF- β 1-dependent ECM protein synthesis. We showed that ERK and JNK activity quickly increase after the interaction between the extracellular HSP70 with TLR-4 determining AP-1 activation.

This transcription factor increased the activity of the TGF- β 1 promoter, leading to increased mRNA as soon as 2 h and supernatant secreted protein levels at 4 h. As a consequence of the quick TGF- β 1 presence, a rapid fibronectin and type I collagen increase in SMC occurred. These quick responses are consistent with the increased TGF- β 1 synthesis observed in pro-fibrotic animal models (Chamberlain et al., 2001; Majesky et al., 1991). In these models, vascular SMCs significantly enhanced TGF- β 1 production and subsequent fibronectin and collagen levels. These changes occur as soon as 2 h after injury intervention, indicating that the cytokine TGF- β 1 responds very quickly.

Our results suggest a novel mechanism in the maintenance of tissue damage in cardiovascular disease. The characteristic vascular cell injury in these processes stimulated the increased release of HSP70 into the extracellular compartment, as observed in atherosclerosis (Pockley et al., 2003). Once the protein is in the circulation, it may interact through TLR4 on the vascular SMCs, stimulating the synthesis of pro-inflammatory cytokines, such as TGF- β 1, leading the deposition of new and abnormal ECM proteins and finally inducing structural changes in different tissues.

Conflict of interest statement

No conflicts of interest, financial or otherwise, are declared by the authors.

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