

## Role of heat shock protein 27 phosphorylation in migration of vascular smooth muscle cells

Hai-Feng Chen · Liang-Di Xie · Chang-Sheng Xu

Received: 13 November 2008 / Accepted: 15 January 2009 / Published online: 4 February 2009  
© Springer Science+Business Media, LLC. 2009

**Abstract** *Objective* The aim of the present study was to investigate the role of heat shock protein 27 (HSP27) phosphorylation in the migration of vascular smooth muscle cells (VSMCs) induced by angiotensin II (AngII) and platelet derived growth factor-BB (PDGF-BB). *Methods* The activity of HSP27 was evaluated by Western blot with specific phospho-HSP27 antibody. F-actin polymerization was detected by FITC-Phalloidine staining using confocal microscopy. Modified Boyden chamber technique was employed for VSMCs migration assessment. *Results* The phosphorylation of HSP27 was induced by AngII and PDGF-BB in a time- and concentration-dependent manner in VSMCs, which was significantly blocked by the HSP inhibitor Quercetin in a concentration-dependent manner. Reorganization of actin stimulated by AngII and PDGF-BB was markedly inhibited by pretreatment with 100  $\mu\text{mol/l}$  Quercetin. The migration of VSMCs induced by AngII and PDGF-BB was partially inhibited by Quercetin with peak inhibition concentration at 100  $\mu\text{mol/l}$ . *Conclusions* HSP27 phosphorylation plays an important role in mediating the rearrangement of F-actin and migration of VSMCs induced by AngII and PDGF-BB. HSP27 may be a potential target for the interventional treatment of pathological process related to cell migration.

**Keywords** Heat shock protein 27 · Cytoskeleton · Cell migration · Vascular smooth muscle cells

### Introduction

Migration of vascular smooth muscle cells (VSMCs) has been suggested to be a key process in the early initiation of lesions of atherosclerosis and restenosis after percutaneous coronary intervention (PCI). Cell migration is believed to be promoted by growth factors and vascular bioactive peptides. However, complete inhibition of the mainstream pathways of intracellular signalling for a specific factor could not fundamentally prevent focus forming, and blockade of a mainstream intracellular signalling for some factors may also inhibit cell migration induced by other chemostimulants believed by another intracellular pathway. There may be a common final pathway key confluence, mediating different active stimulants, contributed to the migration of VSMCs and formation of atherosclerotic plaque. It has been a research orientation toward effectively preventing the VSMCs proliferation-associated diseases by regulating the key gene of such final common paths in cell migration.

Recent reports from several groups suggested that heat shock protein 27 (HSP27) might be a sole effector of actin remodeling [1–4]. Phosphorylation of HSP27 plays an important role in modulating its structural and functional coupling. Nonphosphorylated monomeric HSP27 binds to F-actin as a capping protein and thereby blocks actin polymerization, whereas phosphorylated HSP27 does not affect actin polymerization. Phosphorylation of HSP27 was shown to be necessary for F-actin formation, stabilization of focal adhesions, and promotion of cell migration induced by growth factor stimulation [5–7]. As a result, it

---

H.-F. Chen (✉) · L.-D. Xie (✉) · C.-S. Xu  
Fujian Hypertension Research Institute, First Affiliated Hospital,  
Fujian Medical University, Fuzhou 350005, China  
e-mail: drchf@medmail.com.cn

L.-D. Xie  
e-mail: ldxie@medmail.com.cn

H.-F. Chen  
Department of Cardiology, Fujian Provincial Clinical College,  
Fujian Medical University, Fuzhou 350001, China

is potential that regulating the activity of HSP27 be a therapeutic target for inhibition of cell migration.

The present study was designed to access the role of HSP27 phosphorylation in the migration of VSMCs induced by angiotensin II (AngII) and platelet derived growth factor-BB (PDGF-BB).

## Methods

### Materials

Platelet derived growth factor-BB was from PeproTech. AngII, Quercetin and phalloidin-FITC were obtained from Sigma-Aldrich Co. Phospho-HSP27 antibody was purchased from Alexis. Total-HSP27 antibody and immunoblotting chemiluminescence kit were provided from Santa Cruz Biotechnology. All other reagents were of analytic grade.

### Cell culture

Vascular smooth muscle cells were isolated from thoracic aortas of 8-week-old spontaneously hypertensive rats (SHR) by explant technique and cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 15% fetal bovine serum, 100 kU/l benzylpenicillin, 100 mg/l streptomycin. And the procedures of animal manipulation were approved by the Animal Care and Use Committee of Fujian Medical University. The cells showed a spindle-shaped appearance, and confluent VSMCs in culture showed the characteristic of "hill and valley" growth pattern. The identification of VSMCs was performed by smooth muscle  $\alpha$ -actin staining. Cells were passaged by trypsinization and cells between passage three and five were used for experiments.

### Western blotting

The cells were washed twice with ice-cold PBS, and lysed with SDS sample buffer (Tris-HCl 62.5 mmol/l, SDS 2%, glycerol 10%, DTT 50 mmol/l, bromophenol blue 0.1 %, pH 6.8). Solubilized protein samples were centrifuged (12,000g, 15 min, 4°C) and supernatant protein concentration was determined by Bradford protein assay.

Equal amounts of protein were then separated by SDS-PAGE and transferred to nitrocellulose membranes. Non-specific binding was blocked by 5% fat-free milk powder in Tris-buffered saline containing 0.1% Tween-20 (TBST) at room temperature. The membranes were incubated with primary antibodies of mouse anti-human for Phospho-HSP27 (1:70), and goat anti-human for total-HSP27 (1:500) overnight at 4°C. After washing with TBST, the membranes

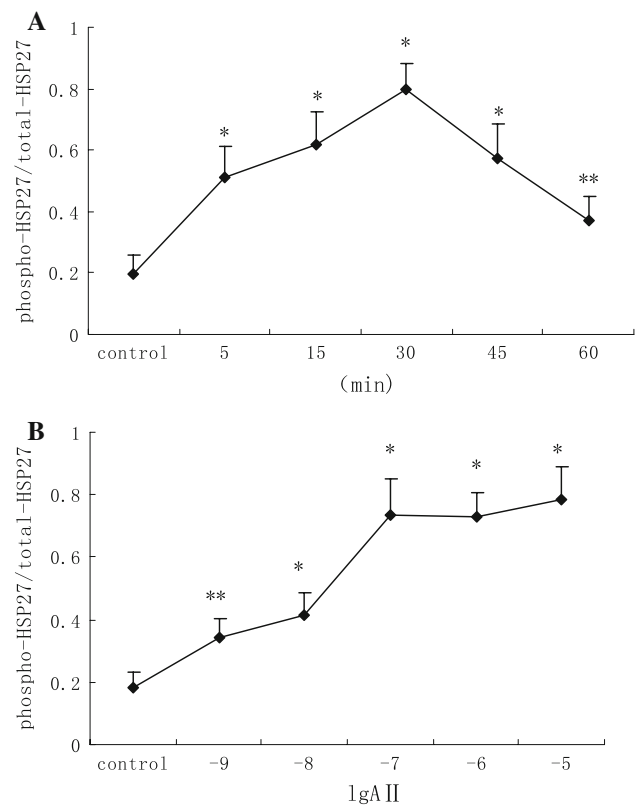
were incubated for 90 min with secondary antibody (goat anti-mouse for Phospho-HSP27, rabbit anti-goat for total-HSP27) at a dilution of 1:2,000 at room temperature. Membranes were then washed three times with TBST and once with TBS. The signals were detected using a chemiluminescence kit and quantified by densitometer analysis.

### Analysis of actin cytoskeleton by confocal microscopy

Vascular smooth muscle cells cultured on glass coverslips were treated with Quercetin for 1 h prior to stimulation with AngII and PDGF-BB for 2 h. Cells were then fixed with 4% paraformaldehyde, permeabilized with 0.1% Triton, and then incubated with Phalloidin-FITC for 60 min in dark. Images of actin filaments were captured and analyzed with a confocal microscopy.

### Cell migration assay

Cell migration was carried out using a modified Boyden chamber. Cells were fasted in serum-free DMEM for 24 h prior to migration experiments. Cells were suspended and counted, and then 0.4 ml was added on the upper chamber



**Fig. 1** Effect of AngII on the phosphorylation of HSP27 in VSMCs. **a** Time-dependent phosphorylation of HSP27 induced by AngII ( $10^{-7}$  mol/l) in VSMCs. **b** Concentration-dependent phosphorylation of HSP27 induced by AngII (30 min). \*\*  $P < 0.05$ , \*  $P < 0.01$  versus control

with a gelatin-coated, polycarbonate membrane (8.0  $\mu\text{m}$  pore) separating two chambers. After 4 h, cells on the upper side of the membrane were scraped using a cotton swab. Cells that migrated to the lower side of the membrane were fixed with 95% alcohol and stained with 0.5% toluidine. The number of migrated cells on the lower side of the filter was counted in five fields under 10 $\times$  magnification. Assays were conducted in duplicate and repeated six times using cultured cells from different animals ( $n = 6$ ).

#### Statistical analysis

Values were expressed as mean  $\pm$  SD, and assessed by one-way ANOVA test.

## Results

### Effect of AngII on activity of HSP27 in VSMCs

The basal level of HSP27 phosphorylation was low in quiescent VSMCs. AngII ( $10^{-7}$  mol/l) induced a rapid,

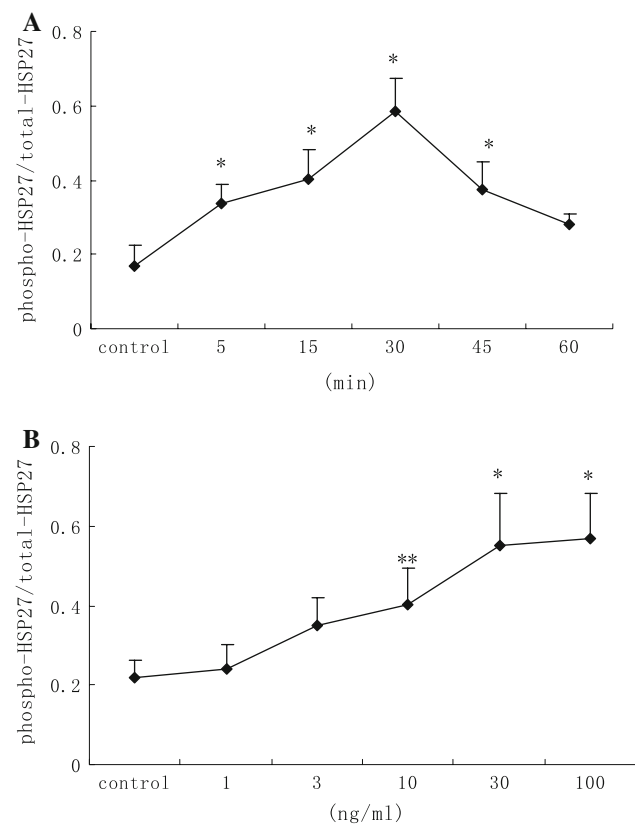
transient increase of HSP27 phosphorylation, with a peak at 30 min. The phosphorylation of HSP27 induced by AngII was increased in a concentration-dependent manner in VSMCs. The phosphorylation of HSP27 in response to  $10^{-5}$  mol/l AngII stimulation was increased by 338.9% compared with control ( $P < 0.01$ ) (Fig. 1).

### Effect of PDGF-BB on activity of HSP27 in VSMCs

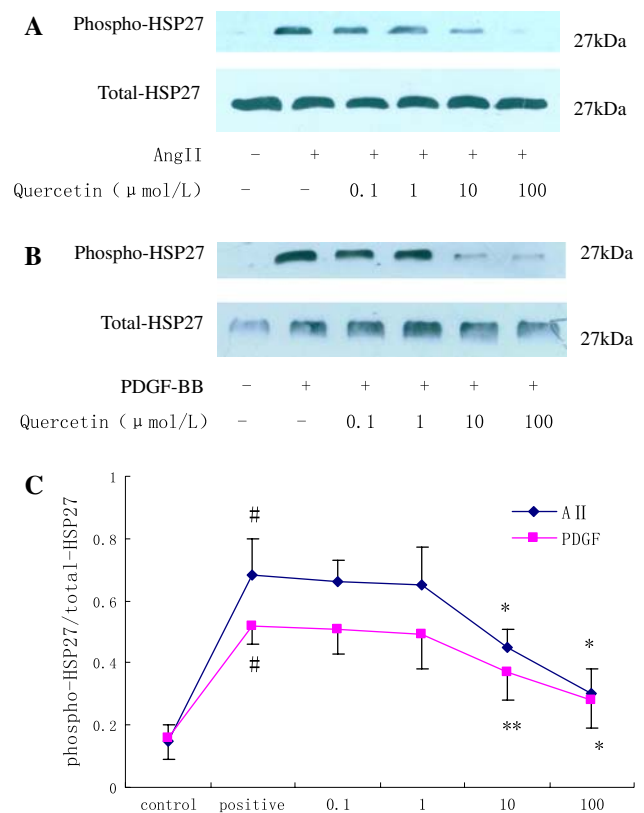
The peak of HSP27 phosphorylation stimulated by PDGF-BB (30 ng/ml) was at 30 min. PDGF-BB induced HSP27 phosphorylation in a concentration-dependent manner in VSMCs. The phosphorylation of HSP27 in response to 100 ng/ml PDGF-BB was increased by 159.1% compared with control ( $P < 0.01$ ) (Fig. 2).

### Effect of Quercetin on activity of HSP27 induced by AngII and PDGF-BB in VSMCs

The phosphorylation of HSP27 induced by AngII and PDGF-BB was blocked by the selective HSP inhibitor Quercetin in a concentration-dependent manner, with

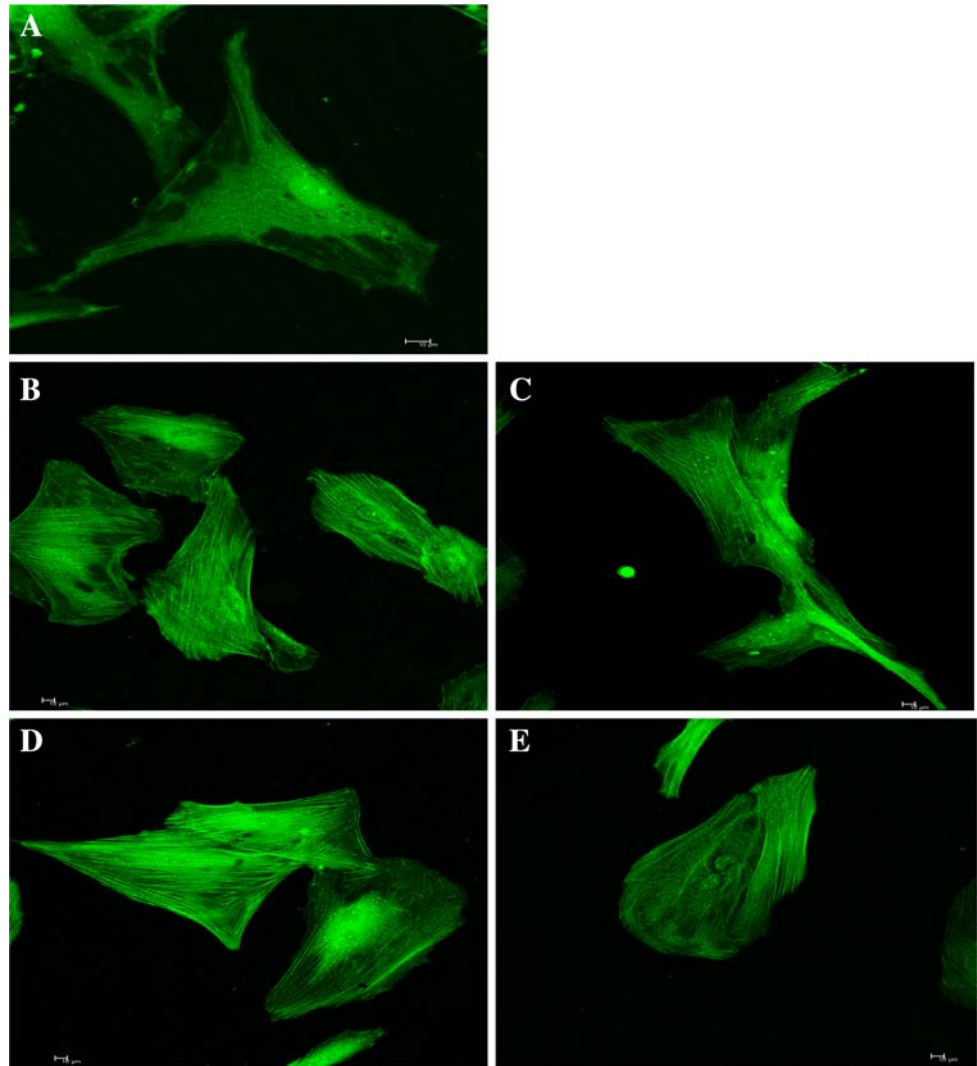


**Fig. 2** Effect of PDGF-BB on the phosphorylation of HSP27 in VSMCs. **a** Time-dependent HSP27 phosphorylation of cultured VSMCs induced by PDGF-BB (30 ng/ml). **b** Concentration-dependent phosphorylation of HSP27 induced by PDGF-BB (30 min) in VSMCs. \*\*  $P < 0.05$ , \*  $P < 0.01$  versus control



**Fig. 3** Concentration-dependent inhibitory effects of Quercetin on the activity of HSP27 induced by AngII ( $10^{-7}$  mol/l, 30 min) and PDGF-BB (30 ng/ml, 30 min) in VSMCs. **a** and **b** autoradiogram; **c** quantitation of immunoreactive bands. #  $P < 0.01$  versus control. \*\*  $P < 0.05$ , \*  $P < 0.01$  versus positive

**Fig. 4** Inhibitory effects of Quercetin (100  $\mu\text{mol/l}$ ) on reorganization of actin cytoskeleton induced by AngII ( $10^{-6}$  mol/l) and PDGF-BB (10 ng/ml) for 2 h. The cells were fixed, stained with FITC-labeled phalloidin and examined by confocal microscopy. **a** control, **b** AngII, **c** AngII + Quercetin, **d** PDGF-BB, **e** PDGF-BB + Quercetin



maximal inhibition rate at 71.7% and 66.7%, respectively ( $P < 0.01$ ) (Fig. 3).

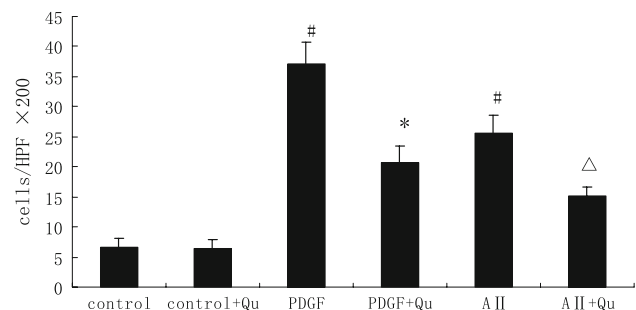
#### Effect of Quercetin on F-actin induced by AngII and PDGF-BB in VSMCs

Treatment with AngII and PDGF-BB resulted in a substantial increase in the number of stress fibers and rearrangement of these structures into ordered parallel arrays in cultured SMCs. Reorganization of actin cytoskeleton stimulated by AngII and PDGF-BB was inhibited by 100  $\mu\text{mol/l}$  Quercetin pretreatment (Fig. 4).

#### Effect of Quercetin on the migration of VSMCs induced by AngII and PDGF-BB

The migration of VSMCs was promoted by AngII and PDGF-BB. Migration of VSMCs was significantly inhibited

by Quercetin. The inhibition rates of 100  $\mu\text{mol/l}$  Quercetin on migration of VSMCs induced by AngII and PDGF-BB were at 55.3% and 53.6% ( $P < 0.01$ ) (Fig. 5).



**Fig. 5** Inhibitory effects of Quercetin (100  $\mu\text{mol/l}$ ) on migration of VSMCs in response to AngII ( $10^{-6}$  mol/l) and PDGF-BB (10 ng/ml) for 4 h. The migrated cells were fixed in 95% alcohol, stained with 0.5% toluidine and counted with a light microscopy at 200 $\times$  magnification. <sup>#</sup>  $P < 0.01$  versus control, <sup>\*</sup>  $P < 0.01$  versus PDGF, <sup>Δ</sup>  $P < 0.01$  versus AII

## Discussion

In this study, we showed that there was basic expression of HSP27 phosphorylation in VSMCs of SHR *in vitro*. AngII and PDGF-BB induced increasing expression of HSP27 phosphorylation in VSMCs in a time- and dose-dependent manner. Pretreatment of Quercetin may inhibit this inducible effect and migration of VSMCs.

Cell migration involves numerous spatially and temporally coordinated cellular processes and occurs in four steps: the formation of actin-rich protrusions such as lamellipodia, cell adhesion, the translocation of the cell body, and rear detachment. There is growing evidence that actin polymerization and the dynamic reorganization of the actin cytoskeleton play important roles in the regulation of cell migration.

HSP27, with a molecular weight of approximately 27 kDa, has been shown to form large aggregates of up to 800 kDa in the cytosol. Expression of HSP27 is up-regulated during the stress response, and correlated with increased survival ability in cells exposed to cytotoxic stimulus. It is a molecular chaperone with an affinity to interact with a large number of different proteins. It has been shown to prevent cell death caused by a variety of toxic agents that promote apoptosis. In addition to enhancing stress tolerance, HSP27 plays a key role in the arrangement of microstructure of actin, one of the most important cytoskeleton component, that is regulated by means of phosphorylation and dephosphorylation [8–10]. Purified unphosphorylated HSP27 homologs from mouse and chicken prevent actin from polymerization *in vitro*. Phosphorylation of HSP27 reverses the inhibition, presumably favoring formation of F-actin *in vivo*. Thus, HSP27 phosphorylation would be expected to increase the dynamics of actin assembly, which is necessary for VSMCs migration [11–13].

Previous reports have shown that the p38 mitogen-activated protein kinase (p38MAPK) pathway, through the activation of downstream kinases MAPK activated protein (MAPKAP) and p38-regulated/activated protein kinase, mediates the phosphorylation of HSP27 [14–20]. Takenaka et al. [19] reported that PDGF-BB phosphorylates HSP27 at Ser-15 and -85 via p38MAPK in cardiac myocytes. Meloche et al. [20] suggested that AngII, working through the AT<sub>1</sub> receptor subtype, stimulated the enzymatic activity of p38, which then promoted the activation of MAPKAP kinase-2 and the phosphorylation of HSP27. Most importantly, incubation with the p38MAPK inhibitor SB203580 reduced the contractile effect of AngII on fresh rat aortic rings. The main finding of the present study is that AngII and PDGF-BB stimulate the phosphorylation of HSP27 in a time- and dose-dependent manner in VSMCs.

To determine whether HSP27 phosphorylation plays roles in the process of the actin reorganization and

migration of VSMCs, we examined the effect of Quercetin on the phosphorylation of HSP27 and cell migration. Quercetin, a bioflavonoid extracted from ginkgo biloba, known as an inhibitor of heat shock factor protein 1 (HSF1), can block Hsps at the transcriptional levels. Our study demonstrated that the phosphorylation of HSP27 induced by AngII and PDGF-BB was partially blocked by Quercetin in a concentration-dependent manner. Reorganization of actin cytoskeleton stimulated by AngII and PDGF-BB was inhibited in 100  $\mu$ mol/l Quercetin pretreatment. So we propose that the phosphorylation of HSP27 is an important pathway that contribute to the rearrangement of F-actin and migration of VSMCs in response to AngII and PDGF-BB.

In summary, our study clearly demonstrated that HSP27 phosphorylation plays a key role in actin filament remodeling and migration of VSMCs in response to AngII and PDGF-BB. HSP27 may be a potential target for the treatment of pathological process related to cell migration.

**Acknowledgment** This work was supported by grant from Key Subjects Development Fund from Fujian Medical University (FJGXY04003).

## References

1. Tartakover-Matalon S, Cherepnin N, Kuchuk M et al (2007) Impaired migration of trophoblast cells caused by simvastatin is associated with decreased membrane IGF-I receptor, MMP-2 activity and HSP27 expression. *Hum Reprod* 22(4):1161–1167. doi:10.1093/humrep/del464
2. Nadin SB, Vargas-Roig LM, Drago G et al (2007) Hsp27, Hsp70 and mismatch repair proteins Hmlh1 and Hmsh2 expression in peripheral blood lymphocytes from healthy subjects and cancer patients. *Cancer Lett* 252(1):131–146. doi:10.1016/j.canlet.2006.12.028
3. Jog NR, Jala VR, Ward RA et al (2007) Heat shock protein 27 regulates neutrophil chemotaxis and exocytosis through two independent mechanisms. *J Immunol* 178(4):2421–2428
4. Lee CK, Lee HM, Kim HJ et al (2007) Syk contributes to PDGF-BB-mediated migration of rat aortic smooth muscle cells via MAPK pathways. *Cardiovasc Res* 74(1):159–168. doi:10.1016/j.cardiores.2007.01.012
5. Purushothaman KR, Meerarani P, Moreno PR (2007) Inflammation and neovascularization in diabetic atherosclerosis. *Indian J Exp Biol* 45(1):93–102
6. Somara S, Bitar KN (2004) Tropomyosin interacts with phosphorylated HSP27 in agonist-induced contraction of smooth muscle. *Am J Physiol Cell Physiol* 286:C1290–C1301. doi:10.1152/ajpcell.00458.2003
7. Chen Y, Currie RW (2006) Small interfering RNA knocks down heat shock factor-1(HSF-1) and exacerbates pro-inflammatory activation of NF- $\kappa$ B and AP-1 in vascular smooth muscle cells. *Cardiovasc Res* 69:66–75. doi:10.1016/j.cardiores.2005.07.004
8. An SS, Fabry B, Mellema M et al (2003) Role of heat shock protein 27 in cytoskeletal remodeling of the airway smooth muscle cell. *J Appl Physiol* 96:1701–1713. doi:10.1152/jappphysiol.01129.2003
9. Hirade K, Tanabe K, Niwa M et al (2005) Adenylyl cyclase-cAMP system inhibits thrombin-induced HSP27 in vascular



- smooth muscle cells. *J Cell Biochem* 94:573–584. doi:[10.1002/jcb.20309](https://doi.org/10.1002/jcb.20309)
10. Suga H, Nakajima K, Shu E et al (2005) Possible involvement of phosphatidylinositol 3-kinase/Akt signal pathway in vasopressin-induced HSP27 phosphorylation in aortic smooth muscle A10 cells. *Arch Biochem Biophys* 438:137–145. doi:[10.1016/j.abb.2005.04.002](https://doi.org/10.1016/j.abb.2005.04.002)
  11. Ying Han, Liangdi Xie, Changsheng Xu (2003) Effect of fluvastatin on migration of vascular smooth muscle cells derived from spontaneously hypertensive rats. *Am J Hypertens* 16(5,S1): A185
  12. Liangdi Xie, Ying Han, Huajun Wang (2004) Effect of ferulate on attachment and migration induced by fibronectin and fibrinogen in cultured vascular smooth muscle cells from shr. *Am J Hypertens* 17(5, S1):s174
  13. Cao H, Dronadula N, Rizvi F et al (2006) Novel role for STAT-5B in the regulation of Hsp27-FGF-2 Axis facilitating thrombin-induced vascular smooth muscle cell growth and motility. *Circ Res* 98:913–922. doi:[10.1161/01.RES.0000216954.55724.a2](https://doi.org/10.1161/01.RES.0000216954.55724.a2)
  14. Pichon S, Bryckaert M, Berrou E (2004) Control of actin dynamics by p38 MAP kinase-HSP27 distribution in the lamellipodium of smooth muscle cells. *J Cell Sci* 117:2569–2577. doi:[10.1242/jcs.01110](https://doi.org/10.1242/jcs.01110)
  15. Tanabe K, Akamatsu S, Suga H et al (2005) Midazolam suppresses thrombin-induced heat shock protein 27 phosphorylation through inhibition of p38 mitogen-activated protein kinase in cardiac myocytes. *J Cell Biochem* 96:56–64. doi:[10.1002/jcb.20455](https://doi.org/10.1002/jcb.20455)
  16. Nguyen A, Chen P, Cai H (2004) Role of CaMKII in hydrogen peroxide activation of ERK1/2, p38 MAPK, HSP27 and actin reorganization in endothelial cells. *FEBS Lett* 572:307–313. doi:[10.1016/j.febslet.2004.06.061](https://doi.org/10.1016/j.febslet.2004.06.061)
  17. Meier M, King GL, Clermont A et al (2001) Angiotensin AT<sub>1</sub> Receptor stimulates heat shock protein 27 phosphorylation in vitro and in vivo. *Hypertension* 38:1260–1265. doi:[10.1161/hy1201.096573](https://doi.org/10.1161/hy1201.096573)
  18. Chen Y, Ross BM, Currie RW (2004) Heat shock treatment protects against angiotensin II-induced hypertension and inflammation in aorta. *Cell Stress Chaperones* 9:99–107
  19. Takenaka M, Matsuno H, Ishisaki A et al (2004) Platelet-derived growth factor-BB phosphorylates heat shock protein 27 in cardiac myocytes. *J Cell Biochem* 91:316–324. doi:[10.1002/jcb.10717](https://doi.org/10.1002/jcb.10717)
  20. Meloche S, Landry J, Huot J et al (2000) p38 MAP kinase pathway regulates angiotensin II-induced contraction of rat vascular smooth muscle. *Am J Physiol Heart Circ Physiol* 279:H741–H751