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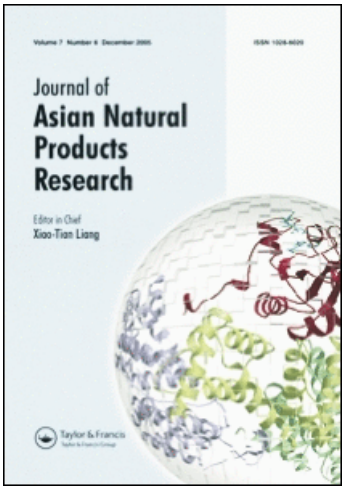

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J. -H. Xiao[a]; Y. -L. Zhang[b]; X. -L. Feng[a]; J. -L. Wang[a]; J. -Q. Qian[a]
[a] Department of Pharmacology, Tongji Medical College of Huazhong University of Science and Technology, Wuhan, China [b] Stomatology Centre, Tongji Hospital of Huazhong University of Science and Technology, Wuhan, China

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Effects of isoliensinine on angiotensin II-induced proliferation of porcine coronary arterial smooth muscle cells

J.-H. XIAO†, Y.-L. ZHANG‡, X.-L. FENG†, J.-L. WANG†* and J.-Q. QIAN†

†Department of Pharmacology, Tongji Medical College of Huazhong University of Science and Technology, Wuhan 430030, China
‡Stomatology Centre, Tongji Hospital of Huazhong University of Science and Technology, Wuhan 430030, China

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The inhibitory effects of isoliensinine (IL), a bisbenzylisoquinoline alkaloid extracted from the seed embryo of the traditional chinese medicinal herb *Nelumbo nucifera Gaertn*, on the proliferation of porcine coronary arterial smooth muscle cells (CASMCs) induced by angiotensin II(Ang II) and its mechanisms of action were investigated. Counting cultured cell number, MTT assay, immunohistochemical method and Western blot were adopted. Ang II 0.1 μmol l^{-1} significantly evoked CASMC proliferation by 42%, which could be dose-dependently inhibited by IL 0.01–3 μmol l^{-1} and the percentage of inhibition of IL 0.1 μmol l^{-1} was 25%. Irbesartan (Irb) 0.1 μmol l^{-1} inhibited CASMC proliferation by 22%. IL or Irb 0.1 μmol l^{-1} decreased Ang II-induced overexpression of Platelet-derived growth factor (PDGF)-β and basic fibroblast growth factor (bFGF), respectively. Both of them also declined c-*fos*, c-*myc* and hsp70 overexpression, respectively. At the same concentration, the inhibitory effects of IL on PDGF-β were even stronger than those of Irb ($P < 0.05$). In summary, the data showed that IL possesses an anti-proliferative effect, which is related to the decrease of the overexpression of growth factors PDGF-β, bFGF, proto-oncogene c-*fos*, c-*myc* and hsp70.

Keywords: Isoliensinine; Angiotensin II; Coronary arterial smooth muscle cell; Proliferation; Growth factor; Proto-oncogene; Hsp70

1. Introduction

Isoliensinine (IL) is a bisbenzylisoquinoline alkaloid extracted from the seed embryo of the traditional Chinese medicinal herb *Nelumbo nucifera Gaertn* and its chemical structure is shown in figure 1 [1]. Previous work from our laboratory has demonstrated that IL possesses an anti-hypertensive effect, anti-hypertrophic effect on left ventricular hypertrophy induced by banding rat abdominal aorta and an anti-arrhythmic effect [2,3]. Its analogues such as liensinine and neferine have been shown to exert anti-proliferatory effects on vascular smooth muscle cells (VSMCs) [4]. These prompted us to consider whether IL might afford an inhibitory effect on proliferation of porcine coronary smooth muscle cells (CASMCs).

*Corresponding author. E-mail: wangjialingtjmu@yahoo.com.cn

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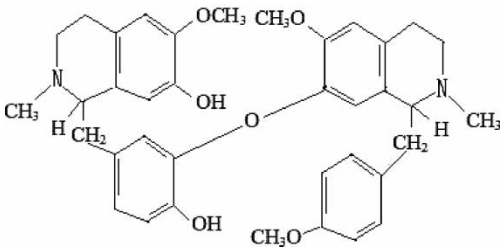

Figure 1. Chemical structure of isoliensinine (IL).

Angiotensin II (Ang II) is the main effective peptide of the renin–angiotensin system, which plays an important role in several cardiovascular diseases associated with VSMC overgrowth and inflammation, including hypertension, atherosclerosis and myocardial infarction [5]. Ang II has been shown to stimulate the proliferation of VSMCs and *trans*-activate platelet-derived growth factor (PDGF)-β, basic fibroblast growth factor (bFGF) [6,7] and enhance expression of c-*fos*, c-*myc* and hsp70 that are related to its proliferative effects on VSMCs [8].

This paper deals with the anti-proliferative effects of IL on porcine CASMCs induced by Ang II and its related mechanisms such as PDGF-β, bFGF, c-*fos*, c-*myc* and hsp70.

2. Results and discussion

2.1 Growth characteristics of porcine CASMCs

Porcine CASMCs exhibited hill and valley patterns at confluence under the optical microscope. Expression of α-smooth muscle actin, regarded as the main characteristic of smooth muscle cells, was detected in cytoplasm of almost all cells cultured by immunohistochemical methods (purity $>99\%$). According to the growth curve, CASMCs went into logarithmic growth phase during 48–72 h.

2.2 Effects of IL on Ang II-induced proliferation of porcine CASMCs

To examine the inhibitory effects of IL on porcine CASMC proliferation, cells were exposed to Ang II with or without IL or Irb for 24 h. Ang II 0.1 μmol l^{-1} significantly promoted the proliferation of CASMCs by 42%, and its effects were attenuated by IL 0.03–3 μmol l^{-1} in a dose-dependent manner. Irb 0.1 μmol l^{-1} as the positive control drug inhibited the proliferation-promoting effects of Ang II on CASMCs by 22%. Moreover, there was no significant difference between the anti-proliferative effects of Irb 0.1 μmol l^{-1} and IL 0.1 μmol l^{-1} (percentage of inhibition = 25%, $P > 0.05$) (table 1).

2.3 Effects of IL on overexpression of growth factors PDGF-β and bFGF in the proliferating CASMCs

The cells were exposed to Ang II 0.1 μmol l^{-1} for 24 h. As shown in figure 2 and table 2, Ang II promoted expression of both PDGF-β and bFGF. Absorbance values were raised from 0.196 ± 0.030 and 0.183 ± 0.035 (as control) to 0.278 ± 0.047 and 0.278 ± 0.021, respectively. Irb or IL 0.1 μmol l^{-1} significantly inhibited their overexpression. Compared

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Table 1. Effect of irbesartan (Irb) and isoliensinine (IL) at different concentrations on the proliferation of porcine CASMCs induced by Ang II (0.1 μmol l^{-1}).

Group	*Concentration (μmol l^{-1})*	*Absorbance (OD)*
Control		0.297 ± 0.017
Ang II	0.1	0.416 ± 0.058**
Irb	0.1	0.327 ± 0.034#
IL	0.03	0.389 ± 0.041
	0.1	0.337 ± 0.037#
	0.3	0.324 ± 0.051##
	1	0.349 ± 0.020#
	3	0.259 ± 0.036##

$\bar{x} \pm s$. $^{**}P < 0.01$ *vs* control. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ *vs* Ang II.

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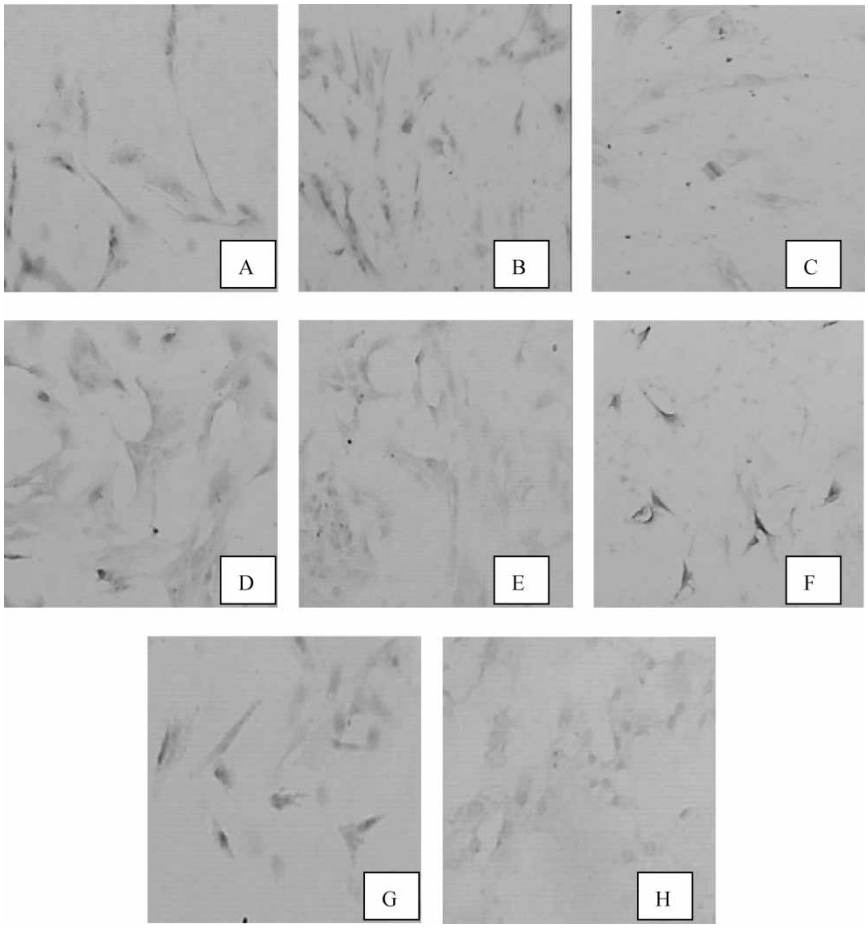

Figure 2. Effects of isoliensinine (IL) on expression of PDGF and bFGF of proliferative CASMCs evoked by Ang II(0.1 μmol l^{-1}). A–D Immunohistochemical photomicrograph of expression of PDGF in CASMCs (10 × 10). (A) control group; (B) AngIIgroup; (C) Irb group; (D) IL group. (E–H) Immunohistochemical photomicrograph of expression of bFGF in CASMCs (10 × 4). (E) control group; (F) Ang II group; (G) Irb group; (H) IL group.

Table 2. Effects of isoliensinine (IL) on expression of PDGF-β and bFGF of proliferative CASMCs evoked by Ang II (0.1 μmol l^{-1}).

Group	*Absorbance*	
	PDGF-β	*bFGF*
Control	0.196 ± 0.030	0.183 ± 0.035
Ang II	0.278 ± 0.047**	0.278 ± 0.021**
Irb	0.219 ± 0.032$^{\#\#}$	0.186 ± 0.035$^{\#\#}$
IL	0.193 ± 0.006$^{\#\#\Delta\Delta}$	0.180 ± 0.007$^{\#\#}$

$n = 30$ fields. $\bar{x} \pm s$. $^{**}P < 0.01$ vs control. $^{\#\#}P < 0.01$ *vs* Ang II, $^{\Delta\Delta}P < 0.01$ *vs* Irb.

with Irb, the inhibitory effect of IL on PDGF-β was stronger. Their inhibition on PDGF-β was 21% and 31% respectively.

2.4 Effects of IL on overexpression of proto-oncogenes c-fos and c-*myc* in the proliferating CASMCs

It is well known that protooncogenes c-*fos* and c-*myc* belong to the family of immediate early genes. Generally, The expression of c-*fos* and c-*myc* approached peak value at 30 and 60 min after treatment, respectively. After treatment with Ang II 0.1 μmol l^{-1}, both c-*fos* and c-*myc* expression of the cells was enhanced from 0.329 ± 0.046 and 0.194 ± 0.035 (as control) to 0.378 ± 0.048 and 0.236 ± 0.049, respectively. As shown in figure 3 and table 3, IL and Irb 0.1 μmol l^{-1} remarkably inhibited the overexpression of c-*fos* and c-*myc*, but the inhibitory effect of IL on c-*myc* expression was weaker. Their inhibition on c-*myc* was 20% and 13%, respectively.

2.5 Effects of IL on the protein content of Hsp70 in porcine CASMCs

As shown in figure 4, AngII 0.1 μmol l^{-1} after treatment for 24 h clearly induced hsp70 overexpression from 100% (as control) to 196.8 ± 68.4%. IL 0.1 μmol l^{-1} inhibited hsp70 overexpression induced by Ang II from 196.8 ± 68.4% to 101.9 ± 36.0%. This inhibitory effect of IL on hsp70 was not obviously different to that of Irb (117.2 ± 58.8%).

2.6 Inhibition of Ang II-induced proliferation of CASMCs by IL

Of many factors involved in stimulating proliferation of VSMCs, Ang II has been the focus of considerable interest. It has been reported that Ang II exerts proliferation–promoting activity on cultured VSMCs for 24 h [9]. In this paper, our results also demonstrated that Ang II a the proliferation-promoting effect on porcine CASMCs. Moreover, we found that IL could inhibit AngII-induced proliferation of CASMCs.

2.7 A very important role of PDGF-β, bFGF, c-*fos*, c-*myc* and Hsp70 in the proliferation of smooth muscle cells

The proliferation-promoting mechanism of AngIIis related to the increase of the expression of PDGF-β, bFGF, c-*fos*, c-*myc* and hsp70, which play a very important role in the proliferation and hypertrophy of smooth muscle cells. PDGF-β and bFGF receptors,

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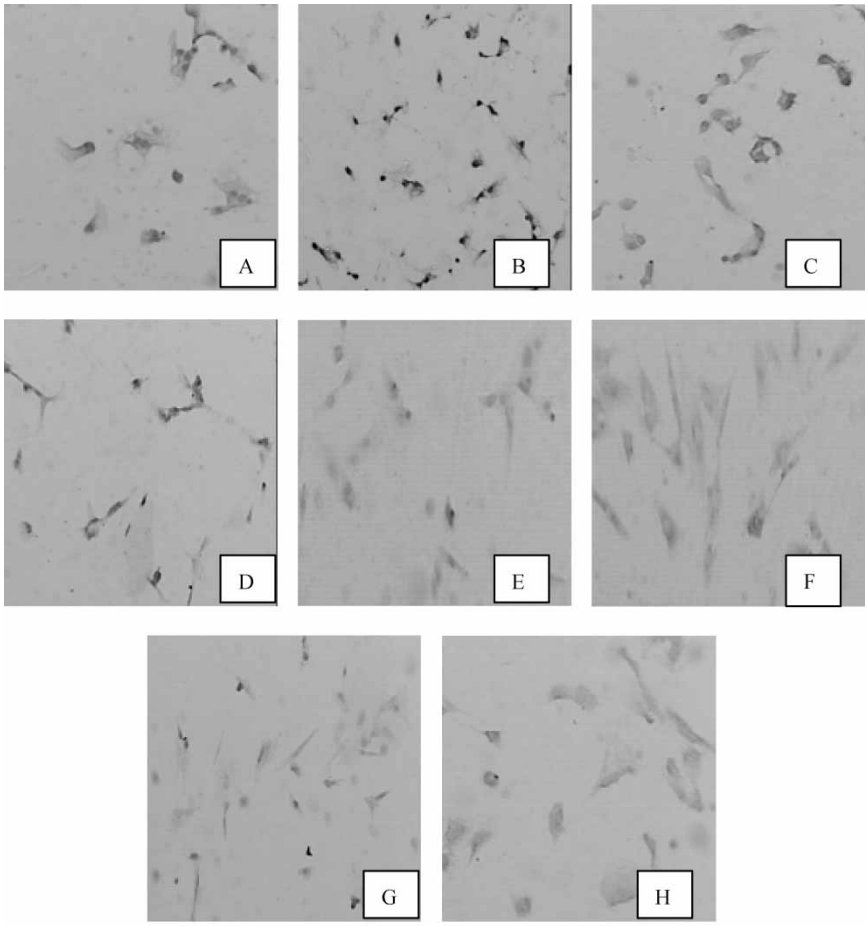

Figure 3. Effects of isoliensinine (IL) on expression of *c-fos* and *c-myc* of porcine proliferative CASMCs induced by Ang II (0.1 μmoll^{-1}). (A–D) Immunohistochemical photomicrograph of *c-fos* expression in CASMCs (10 × 10). (A) control group; (B) AngIIgroup; (C) Irb group; (D) IL group; (E–H) Immunohistochemical photomicrograph of *c-myc* expression in CASMCs (10 × 10). (E) control group; (F) AngIIgroup; (G) Irb group; (H) IL group.

belonging to tyrosine kinase receptors, activate nucleolus transcription factor, regulate expression of nucleolus genes such as c-*fos* and c-*myc,* and cause cell division and proliferation through Ras → Raf → MEK → ERK signal pathways. C-*fos* and c-*myc*, immediately early genes, participate in the processes of cell proliferation, differentiation, signal transduction and apoptosis. Hsp70 is the most important non-specific cell protective

Table 3. Effects of isoliensinine (IL) on expression of *c-fos* and *c-myc* of porcine proliferative CASMCs induced by Ang II (0.1 μmoll^{-1}).

Group	*Absorbance*	
	c-fos	*c-myc*
Control	0.329 ± 0.046	0.194 ± 0.035
Ang II	0.378 ± 0.048**	0.236 ± 0.049**
Irb	0.327 ± 0.048$^{\#\#}$	0.190 ± 0.014$^{\#\#}$
IL	0.331 ± 0.047$^{\#\#}$	0.206 ± 0.029$^{\#\#\Delta\Delta}$

$n = 30$ fields. $\bar{x} \pm s$. $^{**}P < 0.01$ vs control, $^{\#\#}P < 0.01$ *vs* Ang II, $^{\Delta\Delta}P < 0.01$ *vs* Irb.

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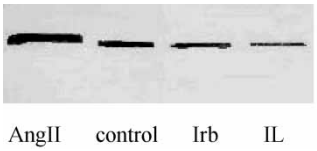

Figure 4. Effect of isoliensinine (IL) on the protein content of hsp70 in porcine CASMCs by Western blot analysis.

protein (molecular chaperone). It combines with receptors and/or protein (tyrosine kinase and serine/threonine kinase) in signalling pathways, then regulates their activity. It takes part in not only important physiological processes such as stress protection, signal transduction, development and differentiation, but also in the pathogenesis of some diseases such as atherosclerosis and tumour [10,11]. The activity of all the above mediators is regarded as intracellular Ca^{2+}- dependence [12,13]. Ang II was able to elevate intracellular Ca^{2+} concentration in VSMC proliferation [14,15]. Hence, a decrease in intracellular Ca^{2+} concentration could reduce their activity.

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2.8 The cause of decreasing expression of PDGF-β, bFGF, c-*fos*, c-*myc* and HSP70 by IL

Although Both IL and Irb inhibited the overexpression of PDGF-β, bFGF, c-*fos*, c-*myc* and hsp70 in proliferating cells, their mode of action was different. Irb, a specific blocker of AT_1 receptors, exerts an effect at the receptor level. IL might not act on the same level. Because the chemical structure of IL is quite different from that of Ang II receptor blockers, IL could non-competitively inhibit aortic contraction induced by AngII (data not shown). In addition, IL could significantly inhibit porcine CASMC proliferation induced by phenylephedrine.

It has been reported that IL possessed a blocking effect on Ca^{2+} influx [3–5]. In another study, the results showed that IL inhibited voltage-dependent L-type Ca^{2+} current and decreased intracellular Ca^{2+} concentration of porcine CASMCs. So the inhibitory effects of IL on expression of c-*fos*, c-*myc* and hsp70 may mainly result from decreasing intracellular Ca^{2+} concentration.

2.9 Conclusion

Our results demonstrated for the first time that IL was able to significantly inhibit Ang II-induced porcine CASMC proliferation. Moreover, IL reversed the overexpression of growth factors PDGF-β, bFGF and proto-oncogenes c-*fos,* c-*myc* and hsp70. Its anti-proliferative effects were almost equal to or even stronger than those of Irb. The findings of the present study may shed light on the pharmacological basis for its clinical application in cardiovascular diseases.

3. Materials and methods

3.1 Drug and reagents

IL (FW = 610.72, purity > 95% by HPLC) was provided by the phytochemistry group of the Department of Pharmacology, Tongji Medical College of Huazhong University of Science and Technology (Chinese patent: ZL 98121706.0, CN 1257070A). It was dissolved

in sterile distilled water and then stored at 4°C, pH 5–6. Dulbecco's modified Eagle's medium (DMEM) and foetal bovine serum (FBS) were purchased from Gibco. Ang II and collagenase I were purchased from Sigma Chemical Co. Irbesartan (Irb) was obtained from Merck. Trypsin was purchased from Difco. Mouse α-smooth muscle actin monoclonal antibody, rabbit PDGF-β polyclonal antibody, rabbit bFGF polyclonal antibody, rabbit c-*fos* polyclonal antibody, mouse hsp70 monoclonal antibody, mouse c-*myc* monoclonal antibody, SABC test kit, DAB test kit and Western blot test kit were purchased from Wuhan Boshide Company. All the other reagents were of analytical grade.

3.2 Porcine CASMC culture

Fresh porcine hearts were obtained from local abattoirs. Coronary arteries sterilely separated from porcine hearts were put into sterilized PBS solution ($g\,l^{-1}$: NaCl 8.0, KCl 0.2, KH_2PO_4 0.2, $Na_2HPO_4{\cdot}12H_2O$ 1.54; pH 7.2–7.4) including 200 U/ml penicillin G and streptomycin. After their fat and artery intima and extima were removed, the arteries were cut into pieces (about 0.5 mm^3) and put into sterilized enzymatic solution (collagenase I 2 mg dissolved in 1 ml DMEM with 10% FBS) for 3–5 h until the tissues became transparent and hairy. The enzymatic solution was maintained at 37°C under regular vibration. Single cells were seeded into sterile culture flasks in DMEM with 10% FBS and incubated at 37°C in humidified 5% CO_2 +95% air. The medium was changed every 72 h. Confluent monolayer cells were established between 12 and 14 days after seeding. Single cell suspension was obtained by 0.25% trypsin. Porcine CASMCs were used from the second to the fourth passage.

3.3 Growth curve of porcine CASMCs

The second-passage cells were seeded in 24-well plates at a density of 2×10^4 cells/well and incubated at 37°C in DMEM with 10% FBS. The cell numbers were counted with a haemocytometer from the first to the seventh day according to conventional methods.

3.4 MTT assay for porcine CASMC proliferation induced by AngII

The cells at a density of 5×10^3 cells/well were seeded in 96-well plates and incubated at 37°C in DMEM with 10% FBS for 24 h. The former medium was replaced with DMEM with 2% FBS. AngII 0.1 μmol/l was simultaneously added to the cells with IL ($0.03–3\ \mu mol/l^{-1}$) or Irb ($0.1\ \mu mol\,l^{-1}$) except the control cells for another 24 h. The anti-proliferative effects of IL were evaluated by MTT assay [16]. The percentage of inhibition of cell growth was derived from the following formula: percentage of inhibition $= (1 - A_{\mathrm{IL/Irb+Ang}}\mathrm{II}/A_{\mathrm{Ang\text{-}II}}) \times 100\%$.

3.5 Immunohistochemical methods for expression of PDGF-β, bFGF, c-*fos* and c-*myc*

The cells were seeded in 24-well plates with slides at a density of 4×10^4 cells/well and incubated in DMEM with 10% FBS at 37°C for 48 h. Then the cells were exposed to DMEM supplemented with 2% FBS. The cells were treated with the drugs for another 24 h, except the groups for detecting the expression of c-*fos* and c-*myc* (c-*fos*: 30 min, c-*myc*: 60 min). Immunoperoxidase staining was performed using the streptavidin–biotin–peroxidase-complex (SABC) method. Immunostaining procedures were carried out according to the

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guidelines of the manufacturer. To determine specificity of immunostaining, control specimens were treated in the same way but without primary antibody.

3.6 Western blot for the protein content of Hsp70

The cells were seeded in 100 ml culture flasks at the same density and incubated in DMEM with 10% FBS at 37°C. The cells were treated with the drugs for another 24 h. The total protein solutions were collected and quantified. Then all solutions were subjected to SDS–polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes as described previously [17]. Hsp70 on the membranes was assayed by antigen–antibody reaction according to the instructions of the Western blot test kit. The immunoreactive bands were visualised by a DAB test kit and quantified by grey degree that was measured by the GeneGenius system (Sygene, USA).

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3.7 Statistical analysis

Data were expressed as $\bar{x} \pm s$ and analyzed using the *t*-test. In all cases, differences were considered significant at $P < 0.05$. Image analysis for PDGF-β, bFGF, c-*fos* and c-*myc* positive cells was performed with HPIAS-1000 image analyser (Tongji Medical College of Huazhong University of Science and Technology, Wuhan, China) at a magnification of 100 ($\times 10$ objective lens). Data were expressed as the absorbance value of positive cells as determined by counting 5 fields per cell slide (6 cell slides per group) under light microscopy. Values are $\bar{x} \pm s$ obtained from 30 fields per group per experiment.

References

[1] J.L. Wang, X.M. Hu, W.H. Yin, H. Cai. *Chin. J. Chin. Mat. Med.*, **16**, 673–675 (1991).
[2] F. Zhang, J.L. Wang, J.Q. Qian. *Chin. Tradit. Herb. Drugs.*, **31**, 753–755 (2000).
[3] X.L. Hong, X. Yu, J.H. Xiao, J.L. Wang, J. Huazhong. *Univ. Sci. Tech. [Health Sci.]*, pp. 608–611 (2002).
[4] J.Q. Qian. *Acta Pharmacol. Sin.*, **23**, 1086–1092 (2002).
[5] K.M. Baker, G.W. Booz, D.E. Dostal. *Annu. Rev. Physiol.*, **54**, 227–241 (1992).
[6] S. Eguchi, H. Iwasaki, T. Inagami, K. Numaguchi, T. Yamakawa, E.D. Motley, et al. *Hypertension*, **33**, 201–206 (1999).
[7] S. Heeneman, J. Haendele, Y. Saito, M. Ishida, B.C. Berk. *J. Biol. Chem.*, **275**, 15926–15932 (2000).
[8] G. Madhu, P.G. Mahesh, A.A. Rene, Z. Radovan. *Prog. Pediatr. Cardiol.*, **9**, 183–197 (1999).
[9] R.M. Youyz, L.Y. Deng, G. He, X.H. Wu, E.L. Schiffrin. *J. Hypertens.*, **17**, 907–916 (1999).
[10] F.U. Hartl. *Nature*, **381**, 571–580 (1996).
[11] S. Lindquist, E.A. Craig. *Ann. Rev. Genet.*, **22**, 631–677 (1988).
[12] T. Shimizu, K. Kinugawa, A. Yao, Y. Sugishita, et al. *Cardiovasc. Res.*, **41**, 641–653 (1999).
[13] R.W. Currie, R.W. Tanguay, J.R. Kingma. *Circulation*, **87**, 963–971 (1993).
[14] N. Macrez-Lepretre, J.L. Morel, J. Mironneau. *J. Pharmacol. Exp. Ther.*, **278**, 468–475 (1996).
[15] R.M. Touyz, F. Tabet, E.L. Schiffrin. *Clin. Exp. Pharmacol. Physiol.*, **30**, 860–866 (2003).
[16] T. Mosmann. *J. Immunol. Methods.*, **65**, 55–63 (1983).
[17] D.E. Smith, P.A. Fisher. *J. Cell. Biol.*, **99**, 20–27 (1984).