BIBLID: ISSN 0253-9756 Acta Pharmacologica Sinica 中国药理学报 1998 Nov; 19 (6): 535 - 540

Inhibitory effects of captopril on c-*myc* expression during left ventricular hypertrophy[1]

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KEY WORDS left ventricular hypertrophy; proto-oncogenes; proto-oncogene proteins c-*myc*; angiotensin Ⅱ; captopril; inbred SHR rats; hypertension; Northern blotting; Western blotting; immunohistochemistry

AIM: To study the molecular mechanism of captopril (Cap) on the inhibition of left ventricular hypertrophy (LVH), disclose the expression and distribution of c-*myc* in different cell types in left ventricle (LV) in spontaneously hypertensive rats (SHR). **METHODS:** Cap 100 mg · kg^{-1} · d^{-1} was given *po* to SHR. Systolic blood pressure (SBP), left ventricular weight (LVW), and body weight (BW) were measured at 16-wk old. The level of angiotensin Ⅱ (Ang Ⅱ), c-*myc* mRNA, and oncoprotein were determined by immunohistochemical method, Northern blot, and Western blot, respectively. **RESULTS:** Cap reduced SBP, LVW/BW in SHR, with a decrease of Ang Ⅱ and c-*myc* expression in LV. Local cardial Ang Ⅱ mainly distributed in cardiomyocytes. Cap inhibited cardial Ang Ⅱ production and c-*myc* expression (histochemical staining intensity index, 0.49 ± 0.04 *vs* 0.83 ± 0.24, $P < 0.01$). The c-*myc* oncoprotein was prevailingly located in cardiac fibroblasts. The c-*myc* oncoprotein in Cap treated SHR was lower than that of WKY. **CONCLUSION:** High expression of c-*myc* in fibroblasts played an important role in the development of LVH in SHR. Inhibitory effects of Cap on LVH was associated with a decreased myocardial Ang Ⅱ and interstitial fibroblasts c-*myc* expression. The c-*myc* oncoprotein post-transcriptional translation was also interrupted by Cap.

Left ventricular hypertrophy (LVH) in essential hypertension is a result of hypertrophy of cardiac myocytes and hyperplasia of cardial fibroblasts along with abnormal accumulation of fibrilar collagen in extracellular space and has been speculated as a response to abnormal expression of a series of hypertension-related genes[1]. Abnormal increasing of local cardial angiotensin Ⅱ (Ang Ⅱ), a result of the activation of cardial renin-angiotensin system (RAS), plays an important role in the pathological process of LVH. *In vitro*, Ang Ⅱ induced an immediately, transiently increased expression of proto-oncogene c-*myc*, which had been supposed to be a common response to mitogenic stimulation[2], in cultured cardiomyocytes and fibroblasts. Increasing c-*myc* expression resulted in hypertrophy in cultured cardiomyocytes and hyperplasia in fibroblasts[3]. The role of c-*myc* in inducing LVH had been also proved by transgenic and antisense oligonucleotide techniques[4]. The previous work[5] in our laboratory has demonstrated that captopril (Cap) could effectively reverse LVH, which is associated with decreased c-*myc* but not c-*fos* proto-oncogene expression in left ventricle in SHR. However, it was yet unknown that the high expression of c-*myc* originated from cardiac myocytes or fibroblasts. This paper was to investigate the exact location of c-*myc* expression in LV, and to disclose the effect of cap on the alteration of c-*myc* expression in various types of cell population in LV.

MATERIALS AND METHODS

Rats Rats were offsprings of breeders derived from Shanghai Institute of Hypertension. Parental ♀ and ♂ adult SHR rats were matched

[1] Project supported by the National Natural Science Foundation of China. No 39270329.

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Received 1996-12-16 Accepted 1998-03-25

in cages, Cap 100 mg·kg^{-1}·d^{-1} was given *po* in solid mixture with small amount milk powder, the ♀ SHR rats were maintained at this dosage throughout pregnancy and lactation. The ♂ weaned pups (n = 28) continued the treatment until 16 wk of age. Only milk was given to sex- and age-matched untreated SHR (n = 27) and WKY controls (n = 28). Experiments were performed on ♂ rats at 16 wk.

Drugs and chemicals Cap was purchased from Sino-American Shanghai Squibb Pharmaceutical Ltd. Chemicals for RNA extraction and diaminobenzidine (DAB) were purchased from Sigma Co. Agarose, random primer kit and T_4 polynucleotide kinase were from Promaga Co (Madison Wi, USA). 3-[*N*-Morpholino] propane-sulfonic acid and nylon membrane were obtained from Boehringer Co. Nitricellulose (NC) membrane was from Hybond^{TM} (Germany), Nuctrap^{TM} Push column from Stratagene Co (La Jolla CA), [α-^{32}P]dCTP and [γ-^{32}P]ATP were from Beijing Furei Co. Rat c-*myc* cDNA probes were obtained from Institute of Cardiovascular Basic Research of Beijing Medical University. 18S oligonucleotide probe was a gift from Dr CHEN Yiu-Fai (University of Alabama, USA). Monoclonal c-*myc* primary antibody was from Santa Cruz Biotechnology, Inc (USA), biotin labeled anti-IgG and SA-HRP were from Vetor Co (USA). Other chemicals were of either AR or molecular biology grade.

Blood pressure (BP) and left ventricular weight (LVW) to body weight (BW) ratio measurement Systolic BP(SBP) was measured using tail-cuff technique (MRB-Ⅲ A computer controlled sphygmomanometer for rats, Shanghai Institute of Hypertension) at 16 wk of age. Rats were weighed before decapitation, the heart was excised, left ventricle including interventricular septum was weighed. LVH was assessed by LVW/BW.

Immunohistochemical study Tissues from LV were fixed in 10 % formaldehyde, paraffin embedded. Sections with 6 μm thick were processed using streptoavidin-peroxidase (SP) immunohistochemical method[6]. Result of immunoreaction was visualized by reaction of horseradish peroxidase (HRP) and diaminobenzidine. Sections were restained with HE and examined under a light microscope (Olympus). Nonspecific staining was ruled out by negative controls (without primary antibody).

RNA extraction and Northern blot Total RNA was prepared[7] from a block of left ventricle (weighed 0.6 to 1.0 g). The quantity and purity of obtained total RNA were determined by measuring the A_{260} and A_{280} at $\lambda_{260\,nm}$ and $\lambda_{280\,nm}$, respectively. Each sample containing the same amount (approximate 50 μg) of total RNA was loaded. Denaturing gel eletrophoresis of RNA was done to confirm the purity of extracted RNA and for further experiments. Then RNA was transferred onto nylon membranes. The membranes were prehybridized in formamide buffer (50 % formamide, 5 × SSPE, 2 × Denhart's buffer, 0.1 % SDS. SSPE: NaCl 7.5 mmol·L^{-1}, $NaH_2PO_4 \cdot H_2O$ 0.55 mmol·L^{-1}, edetic acid 0.1 mmol·L^{-1}, pH 8.0. SDS: sodium lauryl sulfete. Denhart's buffer: 0.02 % Ficoll, 0.02 % polyvinylpyrrolidone, 0.02 % BSA) at 42 ℃ for at least 2 h. Hybridization was performed at 42 ℃ for 24 h using the same buffer containing the appropriciated α-^{32}P labeled c-*myc* cDNA probe prepared by random priming procedure. Unhybridized probe was removed from the nylon membranes with high stringency procedure (2 × SSC/0.1 % SDS at 37 ℃ for 15 min, twice; 0.1 × SSC/0.1 % SDS at 37 ℃ for 15 min, twice; 0.1 × SSC/0.1 % SDS at 55 ℃ for 30 min, twice. SSC: NaCl 150 mmol·L^{-1}, sodium citrate 15 mmol·L^{-1}, pH 7.0). The membranes were exposed to Fuji X-ray Film for 2 - 3 d at -70 ℃. To control the possible samples variability, the membranes were washed in 0.1 × SSC/0.1 % SDS at 95 ℃ for 5 min to remove hybridized radioactive probes. The membranes were rehybridized by [γ-^{32}P]labeled 18S oligonucleotide probe as an internal standard. Relative amount of specific mRNA was determined by densitometric scanner (Beckman Appraise^{TM} Densitometer, USA). The densitometric scores of specific mRNA were normalized by that of 18S rRNA.

Protein extraction and Western blot[8] Briefly, tissue samples, placed in SDS sample buffer (Tris 25 mmol·L^{-1}, NaCl 50 mmol·L^{-1}, 0.5 % sodium deoxycholate, 1 % nonidet P-40, 0.1 % SDS, phenylmethyl sulfonyl fluoride

1 mmol · L^{-1}, approtinin 500 kU · L^{-1}) were homogenized, sonicated, and then centrifuged at 30 000 × g, 4 ℃ for 30 min. The supernatant was boiled for 10 min and protein concentration was measured, then subjected to SDS-PAGE electrophoresis. Samples containing equal 200 μg protein from each rat were loaded per lane. After the transfer of protein onto nitricellulose filters, the filters were incubated for 1 h in phosphate-buffered saline/0.5 % Tween 20 (PBS-T, PBS: NaH_2PO_4 15 mmol · L^{-1}, Na_2HPO_4 80 mmol · L^{-1}, NaCl 1.5 mol · L^{-1}, pH 7.5) containing 5 % nonfat dry milk. Immunological evaluation was performed as followed: filter was incubated at 25 ℃ for 1 h in 1 % BSA/PBS-T buffer containing mouse monoclone antibody against c-*myc* protein 10 mg · L^{-1}, washed with PBS-T and incubated for 30 min with biotin labeled goat anti-mouse IgG antibody, washed again and reacted with streptoavidin again. After extensive washing with PBS-T, the immunocomplexes on the filters were visualized for 1 min with diaminobenzidine (DAB). Finally, the filters were photographed and subjected to densitometric analysis (Beckman Appraise™ Densitometer, USA).

Statistical analysis Data were expressed as $\bar{x} \pm s$. Student-Newman Keuls procedure in Statistical Package for the Social Science (SPSS) was used to evaluate the differences between Cap treated SHR, untreated SHR, and WKY groups.

RESULTS

Cap prevented the development of hypertention and LVH in SHR At 16 wk of age, SBP in Cap treated SHR (n = 28) was lower than that of untreated SHR (n = 27), (18.8 ± 2.6) *vs* (25.7 ± 2.7) kPa, (P < 0.01), but still higher than that of WKY (n = 28), (18.8 ± 2.6) *vs* (16.2 ± 1.8) kPa, (P < 0.01). LVW/BW in SHR was markedly greater than that of WKY (3.6 ± 0.4) *vs* (2.5 ± 0.3) mg · g^{-1}, (P < 0.01). Cap prevented the development of LVH in SHR [LVW/BW: (2.61 ± 0.20) *vs* (3.6 ± 0.4) mg · g^{-1}, P < 0.01] (Tab 1).

Tab 1. Systolic blood pressure (SBP) and left ventricular weight to body weight ratio (LVW/BW) in Cap treated SHR (SHRcap), untreated SHR, and WKY rats at 16-wk old. $\bar{x} \pm s$. $^cP < 0.01$ *vs* SHR. $^fP < 0.01$ *vs* WKY.

	SHR (27 rats)	SHRcap (28 rats)	WKY (28 rats)
SBP, kPa	25.7 ± 2.7	18.8 ± 2.6^c	16.2 ± 1.8
LVW/BW, mg · g^{-1}	3.6 ± 0.4	2.61 ± 0.20^c	2.5 ± 0.3

Local cardial Ang Ⅱ was mainly produced by cardiomyocytes and was reduced by Cap treatment Immunohistochemical staining showed that cardial Ang Ⅱ was predominantly localized in cardiomyocytes. In SHR, local cardial Ang Ⅱ was apparently higher than that of WKY (histochemical staining intensity index, HSII: 0.83 ± 0.24 *vs* 0.45 ± 0.03, n = 12, P < 0.01). Cap inhibited cardial Ang Ⅱ production of SHR (HSII: 0.49 ± 0.04 *vs* 0.83 ± 0.24, n = 12, P < 0.01) to a level approximate to that of WKY (HSII: 0.49 ± 0.04 *vs* 0.45 ± 0.03, n = 12, P > 0.05) (Fig 1, Plate 1).

Cap inhibited c-*myc* mRNA expression and oncoprotein in LV of SHR Expression of c-*myc* mRNA in LV of SHR was 2.03-fold higher than that of WKY, Cap inhibited c-*myc* expression (arbitary OD units, 0.92 ± 0.17 *vs* 1.69 ± 0.21, n = 8, P < 0.01), but it was still higher than that of WKY (arbitary OD units, 0.92 ± 0.17 *vs* 0.56 ± 0.12, n = 8, P < 0.01) (Fig 2).

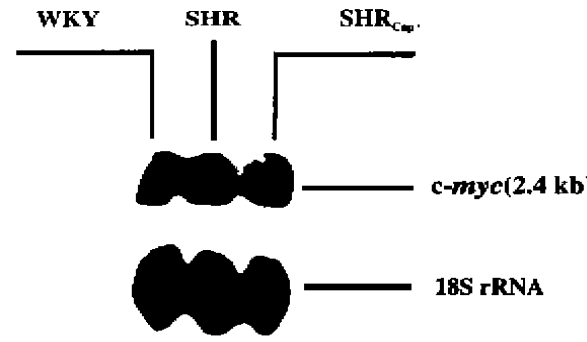

Fig 2. Northern blot showing the expression of proto-oncogene c-*myc* mRNA in LV in SHR, Cap treated SHR (SHRcap), and WKY rats at 16 wk of age. Rehybridization of the same blot with 18S rRNA probe, serving as internal standard, indicated the amount of total RNA was loaded in each lane (bottom). Each group n = 8 rats.

Western immunoblotting was not totally coincident with Northern blot. Oncoprotein of c-*myc* in SHRcap was lower not only than that

of SHR, (OD value: 2.1 ± 0.4 *vs* 20 ± 6, n = 8, $P < 0.01$), but also than that of WKY (OD value, 2.1 ± 0.4 *vs* 10.9 ± 1.4, $n = 8$, $P < 0.01$) (Fig 3).

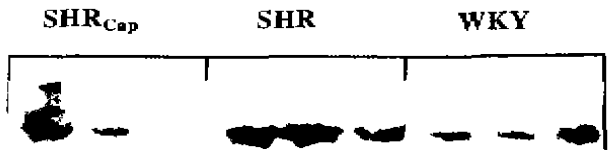

Fig 3. Western blot showing the levels of oncoprotein c-*myc* in LV in SHR, Cap treated SHR (SHRcap), and WKY rats (each group $n = 12$) at 16-wk old. Each lane contained 200 μg total protein.

c-*myc* Oncoprotein mainly located in cardiac fibroblasts c-*myc* Oncoprotein was mainly located in the nuclei of fibroblasts. The HSII of c-*myc* in fibroblasts was 2.61 folds of that in cardiac myocytes (Fig 1, Plate 1, Tab 2).

Tab 2. Oncoprotein c-*myc* in cardiomyocytes and cardiac fibroblasts in LV of Cap treated SHR (SHRcap), untreated SHR and WKY rats at 16-wk old. Results were assessed by histochemical staining intensive index (HSII).
Each group $n = 8$ rats. $\bar{x} \pm s$.
$^cP < 0.01$ *vs* SHR. $^dP > 0.05$, $^fP < 0.01$ *vs* WKY.

	SHR	SHRcap	WKY
Myocytes	1.0 ± 0.3	0.44 ± 0.23^{cd}	0.6 ± 0.4
Fibroblasts	2.61 ± 0.11	0.21 ± 0.10^{cf}	0.53 ± 0.11

Elevated c-*myc* expression, critical to the development of LVH There was a positive correlation between LVW/BW and c-*myc* mRNA ($r = 0.97$, $P < 0.01$) (Fig 4). No correlation showed between SBP and LVW/BW ($r = 0.0335$, $P = 0.93$), or c-*myc* mRNA and SBP ($r = -0.419$, $P = 0.915$).

DISCUSSION

Present study demonstrated the underlying interaction between cardiomyocytes and cardial fibroblasts in promoting LVH and the correlation in the reversal of LVH and depressed expression of proto-oncogene c-*myc* by Cap treatment. We found that cardial Ang Ⅱ was mainly located in cardiomyocytes but little in fibroblasts. This seemed to indicate that cardiomyocytes were more

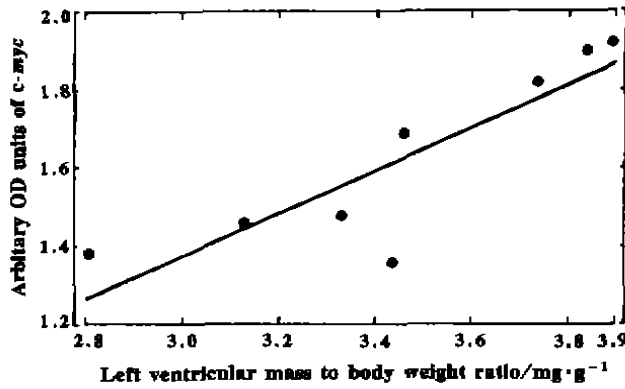

Fig 4. Correlation between left ventricular weight to body weight ratio (LVW/BW) and the arbitary OD units of c-*myc* mRNA expression in LV of SHR ($n = 9$, $r = 0.97$, $P < 0.01$).

critical to the development of LVH than fibroblasts. However, the fact was that collagen usually grew faster than cardiomyocytes in LV of SHR, resulting in fibrosis in LV[9]. Ang Ⅱ functions through angiotensin type 1 receptor (AT_1)[10]. In cardiac fibroblasts, but not in myocytes, there existed high affinity and density of AT_1 receptor in relation to high level of AT_1 receptor mRNA expression in neonatal rat heart[11]. The different distribution in Ang Ⅱ and its functional receptor between fibroblasts and cardiomyocytes suggested a potential role for the fibroblasts in mediating the effect of Ang Ⅱ in the heart. The proliferation of cardiac fibroblasts and subsequential collagen synthesis may be resulted from Ang Ⅱ stimulation paracrined by cardiac myocytes. Increasing Ang Ⅱ in LV, on the other hand, inhibited the activity of collagenase, or matrix metalloproteinase, a critical enzyme for degradation of collagen, which eventually increased the accumulation of collagen[12]. A transiently, immediately increasing expression of proto-oncogene c-*myc* was demonstrated as a common response to hormonal and mechanical stimuli[2]. However, our data, together with other investigators[4], demonstrated that well developed LVH in SHR was accompanied by persistant high expression of c-*myc* mRNA. We also confirmed this result at post-transcriptional level and further found c-*myc* oncoprotein was predominately located in the nuclei of cardiac fibloblasts. The mechanism of such different c-*myc* expression styles between

myocytes and nonmyocytes remains obscure. c-*myc* had been reported to be expressed in G_0 phase in cell growth cycle[14]. Impotence in mitosis of postnatal cardiomyocyte may be produced by the loss of stimulating growth factor, or transducer or acquisition inhibitary factors[15], thus limiting cell division and c-*myc* expression. In contrast to cardiomyocytes, cardiac fibroblasts are undergoing constant hyperplasia responding to Ang Ⅱ stimulation resulting in a longlasting expression of c-*myc*.

Cap was effective in the reversal of left ventricular hypertrophy, which was associated with a decreased level of local cardial Ang Ⅱ[14]. Our data showed a significant decrease of Ang Ⅱ content in myocyte and c-*myc* expression in fibroblasts. Decrease in Ang Ⅱ contributed to the regression of hypertrophy in cardiac myocytes and further prevented hyperplasia in fibroblasts. Except for these, the inhibitory effects of Cap on LVH may also benifit from a descendant blood pressure and removal of the inhibition on metalloproteinase by Ang Ⅱ. Surprisingly, c-*myc* oncoprotein was inhibited to a level even lower than that of its normotensive control. The exact mechanism of this phenomenon remains to be further studied.

This study explored an underlying molecular mechanism of LVH confirming that the initial activation of cardial renin-angiotensin system critically triggered LVH. Ang Ⅱ dominantly induced c-*myc* expression in cardial fibroblasts which resulted in heart hypertrophy. Cap effectively inhibited Ang Ⅱ production and subsequent c-*myc* expression, thus preventing the development of LVH.

REFERENCES

1 Yang YD, Li QH, Zhang XB. Advances in identifying the genes relating to essential hypertension. Chin J Hypertens 1993; 1: 1 – 4.

2 Rosenwald IB, Rhoads DB, Callanan LD, Isselbacher KJ, Schmidt EV. Increased expression of eukaryotic translation initiation factors eIF-4E and eIF-2α in response to growth induction by c-*myc*. Proc Natl Acad Sci USA 1993; 90: 6175 – 8.

3 Sadoshima J-i, Izumo S. Molecular characterization of angiotensin Ⅱ-induced hypertrophy of cardiac myocytes and hyperplasia of cardiac fibroblasts: critical role of the AT_1 receptor subtype. Circ Res 1993; 73: 413 – 23.

4 Jackson T, Allard MF, Sreenan CM, Doss LK, Bishop SP, Swain JL. The c-*myc* proto-oncogene regulates cardiac development in transgenic mice. Mol Cell Biol 1990; 10: 3709 – 16.

5 Chen SC, Chen DG, Bao YD, Jin XQ, Lin YQ, Wang HJ. Inhibition of left ventricular hypertrophy and expression of proto-oncogenes c-*myc* other than c-*fos* in myocardium by early captopril treatment in SHR rats. Acta Pharmacol Sin 1995; 16: 217 – 22.

6 Zou XJ, Cai L, Cai MX, Ni CR, Wei YL. Relation between the expression of oncogene *ras* and cell growth cycle in cervical carcinoma cell. J Clin Exp Pathol 1995; 11: 187 – 8.

7 Chomczynski P, Sacchi N. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal Biochem 1987; 162: 156 – 9.

8 Wang JY, Johnson LR. Expression of protooncogenes c-*fos* and c-*myc* in healing of gastric mucosal stress ulcers. Am J Physiol 1994; 266: G878 – 86.

9 Jalil JE, Doering CW, Janicki JS, Pick R, Shroff SG, Weber KT. Fibrillar collagen and myocardial stiffness in the intact hypertrophied rat left ventricle. Circ Res 1989; 64: 1041 – 50.

10 Dostal DE, Baker KM. Angiotensin Ⅱ stimulation of left ventricular hypertrophy in adult rat heart: mediation by AT_1 receptor. Am J Hypertens 1992; 5 (5 Pt 1): 276 – 80.

11 Kim NN, Villarreal FJ, Printz MP, Lee AA, Dillmann WH. Trophic effects of angiotensin Ⅱ on neonatal rat cardiac myocytes are mediated by cardiac fibroblasts. Am J Physiol 1995; 269: E426 – 37.

12 Brilla CG, Maisch B, Zhou G, Weber KT. Hormonal regulation of cardiac fibroblast function. Eur Heart J 1995; 16 Suppl C: 45 – 50.

13 Dani CH, Mechti N, Piechaczyk M, Lebleu B, Jeanteur PH, Blanchard JM. Increased rate of degradation of c-*myc* mRNA in interferon-treated Daudi cells. Proc Natl Acad Sci USA 1985; 82: 4896 – 9.

14 Samuel JL, Dubus I, Contard F, Schwartz K, Rappaport L. Biological signals of cardiac hypertrophy. Eur Heart J 1990; 11 Suppl G: 1 – 7.

15 Hu WY, Chen DG, Chen SC, Jin XQ, Wang HJ. Effect of chronic captopril treatment on circulating and tissue renin-angiotensin system in SHR rats. Acta Pharmacol Sin 1996; 17: 507 – 12.

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卡托普利抑制原癌基因 c-*myc* 在左心室肥大时的表达[1]

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关键词 左心室肥大; 原癌基因类; 原癌基因蛋白 c-*myc*; 血管紧张素Ⅱ; 卡托普利; 近交 SHR 大鼠; 高血压; RNA 印迹; 蛋白质印迹; 免疫组织化学

目的：观察卡托普利(Cap)对原癌基因 c-*myc* 在自发性高血压大鼠(SHR)左心室心肌不同细胞类型的表达的影响，探讨 Cap 抑制左心室肥大的分子机制. 方法：SHR 宫内期口服给药(100 mg·kg^{-1}·d^{-1})，雄性 SHR 16 周龄时测定收缩压、左室重与体重比. 左心室 c-*myc* mRNA，Ang Ⅱ及 c-*myc* 癌蛋白表达量分别用 RNA 印迹、免疫组化及蛋白质印迹测定. 结果：Cap 明显降低 SHR 大鼠的收缩压，抑制左心室肥大及 c-*myc* 表达，心肌局部 Ang Ⅱ主要产生于心肌细胞，c-*myc* 则主要源于成纤维细胞. 结论：Cap 通过抑制心肌细胞的 Ang Ⅱ和成纤维细胞的 c-*myc* 表达而逆转左心室肥大.

BIBLID: ISSN 0253-9756 Acta Pharmacologica Sinica 中国药理学报 1998 Nov; 19 (6): 540 - 542

Effects of genistein on aggregation and cytosolic free calcium in pig platelets[1]

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KEY WORDS isoflavones; genistein; thrombin; platelet aggregation; calcium; protein-tyrosine kinase

AIM: To study the effects of genistein on aggregation and cytosolic free calcium concentration in platelets. **METHODS**: Using turbidimetry to analyse aggregation and using Fura-2 fluorescence technique to determine Ca^{2+} level. **RESULTS**: Genistein strongly inhibited the pig platelet aggregation induced by thrombin (250 U·L^{-1}). When genistein concentrations were 5 and 20 μmol·L^{-1}, the inhibition rates on the aggregation were 52 % and 73 %, respectively. Genistein inhibited the rise of cytosolic free calcium concentration in platelets stimulated by thrombin (500 U·L^{-1}) in the presence of extracellular Ca^{2+} 1 mmol·L^{-1}. When genistein concentrations were 10, 20, 40, and 80 μmol·L^{-1}, the inhibition rates were 24 %, 40 %, 63 %, and 65 %, respectively, but no effect on thrombin-induced internal Ca^{2+} release from dense tubular system. **CONCLUSION**: Genistein is a potential anti-platelet agent, mainly due to an inhibition of Ca^{2+} influx.

Genistein (4,5,7-trihydroxyisoflavone) is a specific inhibitor of tyrosine protein kinase (TPK)[1]. It inhibits platelet shape change and protein tyrosine phosphorylation in platelet[2]. In the present study, we investigated the effect of genistein on thrombin-induced pig platelet aggregation.

MATERIALS AND METHODS

Thrombin, egtazic acid, Fura 2-AM, Triton X-100, bovine serum albumin (BSA), RPMI 1640, and genistein were from Sigma. All other chemicals were AR.

Pig blood collected in plastic tubes and anticoagulated with 0.15 volume of ACD (trisodium citrate 86, glucose 111, citric acid 53 mmol·L^{-1}).

Platelet aggregation Pig blood was spun at 200 × *g* for 15 min, and the supernatant was then spun at 800 × *g* for 10 min. The cells were resuspended at 2 × 10^{11} platelets·L^{-1} in Tyrode-HEPES buffer (NaCl 140, KCl 5, $MgSO_4$ 1, HEPES 10, glucose 10 mmol·L^{-1}, pH 7.4). Platelet aggregometry was carried by an SPA-4 aggregometer (Shanghai). The platelet suspensions (0.2 mL) were incubated with genistein for 2 min, and then stimulated with thrombin 250 U·L^{-1} for 3 min.

Cytosolic free calcium Suspensions of 2 × 10^{11} platelets·L^{-1} in an RPMI-1640 (pH 7.4) containing 0.2 % BSA and HEPES 10 mmol·L^{-1} were incubated with Fura 2-AM 2.5 μmol·L^{-1} at 25 ℃ for 45 min. The cell suspension was then spun at 800 × *g* for 10 min. The resuspension was of 3 × 10^{10} – 4 × 10^{10} platelets·L^{-1} in a Tyrode-HEPES buffer. Fluorescence

[1] Project supported by Guangdong Research Foundation for Key Field, No 9306.
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Received 1998-04-08 Accepted 1998-04-25