

## Downregulation of the Ornithine Decarboxylase/polyamine System Inhibits Angiotensin-induced Hypertrophy of Cardiomyocytes Through the NO/cGMP-dependent Protein Kinase Type-I Pathway

Yan Lin<sup>1,2</sup>, Ji-Cheng Liu<sup>1</sup>, Xiao-Jie Zhang<sup>1</sup>, Guang-Wei Li<sup>1,2</sup>, Li-Na Wang<sup>2</sup>, Yu-Hui Xi<sup>2</sup>, Hong-Zhu Li<sup>2</sup>, Ya-Jun Zhao<sup>2</sup> and Chang-Qing Xu<sup>2,3</sup>

<sup>1</sup>Department of Pathophysiology, Qiqihar Medical University, Qiqihar, <sup>2</sup>Department of Pathophysiology, Harbin Medical University, Harbin, <sup>3</sup>Bio-Pharmaceutical Key Laboratory of Heilongjiang Province, Harbin

### Key Words

Polyamines • Hypertrophy • NO • Cardiomyocyte

### Abstract

**Background:** Polyamines and nitric oxide (NO) have been involved in the pathogenesis of cardiac hypertrophy. NO can regulate cardiac ion channels by direct actions on G-proteins and adenylyl cyclase. The present study was undertaken to elucidate the molecular mechanism of interactions with polyamines and NO in cardiac hypertrophy. **Methods:** Cardiomyocyte hypertrophy was induced by angiotensin II (AngII). Hypertrophy was estimated by cell-surface area, atrial natriuretic peptide (ANP) mRNA expression, and the immunofluorescence of phalloidin. Pretreatment with alpha-difluoromethylornithine (DFMO) was done to deplete putrescine; KT5823 pretreatment was carried out to block the nitric oxide/cGMP-dependent protein kinase type-I (NO/PKG-I) pathway. Expressions of endothelial nitric oxide synthase (eNOS), PKG-I, c-fos and c-myc were analyzed by western blotting and immunofluorescence. The intracellular concentration of free calcium ( $[Ca^{2+}]_i$ ) was determined by confocal laser

scanning microscopy. **Results:** Hypertrophy of cardiomyocytes was induced by AngII, this caused an increase in putrescine, spermidine and total polyamine pool in association with a decreased level of NO. Expressions of eNOS and PKG-I were down-regulated,  $[Ca^{2+}]_i$  was increased, and expressions of c-Fos and c-Myc upregulated. DFMO reversed these changes induced by AngII. **Conclusions:** Downregulation of polyamines inhibits cardiomyocyte hypertrophy, which is closely related to  $[Ca^{2+}]_i$  and the NO/PKG-I pathway.

Copyright © 2010 S. Karger AG, Basel

### Introduction

Cardiac hypertrophy has been viewed as a compensatory mechanism that helps to maintain cardiac output during disease states with sustained increases in hemodynamic load. However, hypertrophy often heralds decompensation, transition to heart failure, and sudden death [1]. To understand the molecular mechanisms underlying this ultimately maladaptive response, studies have focused on the signaling pathways controlling hypertrophy at the level of the single cardiac myocyte (cardiomyocyte). These include specific G-protein isoforms, low-molecular-weight GTPases,  $Ca^{2+}$ -sensitive

signaling pathways and protein kinase C (PKC) [2-4].

It is well known that many stimuli, including growth factors and cytokines, bind to heterotrimeric G-protein-coupled receptors and rapidly activate immediate-early genes (e.g. *c-fos*, *c-jun*, *egr-1*) that encode transcription factors via the PKC pathway and the mitogen-activated protein kinase (MAPK) phosphorylation cascade [5]. Subsequently, late target genes such as atrial natriuretic peptide (ANP),  $\beta$ -myosin heavy chain ( $\beta$ -MHC), skeletal  $\alpha$ -actin, and myosin light chain 2 (normally expressed in the embryonic ventricle), are induced, ultimately leading to cardiomyocyte hypertrophy [6].

Polyamines (spermine, spermidine and putrescine) belong to a family of low-molecular-weight organic polycations known to be important mediators for the growth and division of cells. Polyamines are involved in the progression of the cell through the cell cycle, which is the basis for cell proliferation [7, 8]. Polyamines share a common precursor with nitric oxide (NO). NO is an important intracellular signal molecule. Our previous studies demonstrated that polyamine metabolism is influenced by NO in cardiac hypertrophy, L-arginine can attenuate cardiac hypertrophy through downregulation of polyamine metabolism and upregulation of NO pathways [9].

Studies in several experimental models have suggested that the polyamine and NO pathways are inter-regulated. For example, NO inhibits proliferation of vascular smooth muscle cells by inhibiting the activity of ornithine decarboxylase (ODC, the key enzyme in polyamine biosynthesis) in atherosclerosis [10]. NO and polyamine-dependent pathways are involved in the signal transmission of free radicals in neutrophil nutrient homeostasis [11].

Whether the ODC/polyamine system is involved in inhibition of hypertrophy of cardiomyocytes, and the relationship between the ODC/polyamine system and the nitric oxide/cGMP-dependent protein kinase type-I (NO/PKG-I) pathway, have yet to be determined. We postulated that downregulation of the ODC/polyamine system may suppress hypertrophy via NO-dependent signaling pathways.

## Materials and Methods

The study was approved by our Institutional Animal Research Committee. All animals received humane care in compliance with the Guide for the "Care and Use of Laboratory Animals" (National Institute of Health publication 86-23, revised 1986).

### Materials

Angiotensin II (AngII), difluoromethylornithin (DFMO), fluorescein isothiocyanate (FITC)-conjugated phalloidin, KT5823 and trypsinase were purchased from Sigma-Aldrich (St. Louis, MO, USA). The primary antibodies anti-eNOS, c-Fos and c-Myc were obtained from Santa Cruz Biotechnology Incorporated (Santa Cruz, CA, USA) and anti-PKG-I was from Stressgen Bioreagents Corporation (Ann Arbor, MI, USA). The western blot kit was from Promega (Madison, WI, USA). The NO assay kit was purchased from the Nanjing Jiancheng Bioengineering Institute (Nanjing, China).

### Incubation of neonatal rat cardiomyocytes incubation

Neonatal rat cardiomyocytes were prepared from 2-3-day-old neonatal Wistar rats (Animal Research Institute of Harbin Medical University, Heilongjiang, China). Rats were anesthetized and killed by immersing in 70% (v/v) alcohol. The ventricles were removed and washed thrice in D-Hanks balanced salt solution (g/L: 0.4 KCl, 0.06 KH<sub>2</sub>PO<sub>4</sub>, 8.0 NaCl, 0.35 NaHCO<sub>3</sub> and 0.06 Na<sub>2</sub>HPO<sub>4</sub> 7H<sub>2</sub>O, pH 7.2) at 4°C. They were then minced and incubated with 0.25% (w/v) trypsinase for 10 min at 37°C. Addition of an equal volume of cold Dulbecco's modified Eagle's medium (DMEM) containing 10% (v/v) newborn calf serum was used to terminate the digestion. The supernatant was discarded. Cells were then incubated with fresh 0.25% trypsinase for 15 min at 37°C, and the supernatant was collected. The latter digestion step was repeated four times. Cells in the supernatant were isolated by centrifugation for 10 min at 1500 rpm at room temperature. Cells were resuspended in DMEM containing 20% (v/v) newborn calf serum, 100 U/mL penicillin and 100 mg/mL streptomycin. Cells were then cultured as monolayers at a 5×10<sup>4</sup> cells/cm<sup>2</sup> at 37°C in a humidified atmosphere containing 5% (v/v) CO<sub>2</sub>. The medium contained 2  $\mu$ M fluorodeoxyuridine to prevent proliferation of non-myocytes.

### Experimental protocol

Three days after being seeded, the neonatal rat cardiomyocytes were starved in serum-free DMEM for 24 h. These cardiomyocytes were then randomly divided into five groups: (1) normal control group; (2) AngII group: cardiomyocytes were treated with 100 nM AngII for 48 h; (3) pre-DFMO group: cardiomyocytes were pre-incubated with 0.5 mM DFMO (a specific inhibitor of ODC) for 24 h and then treated with 100 nM AngII for 48 h; (4) pre-(DFMO+Pu) group: cardiomyocytes were pre-incubated with 0.5 mM DFMO and 0.5 mM putrescine for 24 h and then treated with 100 nM AngII for 48 h; and (5) pre-(DFMO+KT5823) group the procedure was similar to that for group 3, except that the cardiomyocytes were pre-incubated with KT5823 (a specific inhibitor of PKG-I) for 1 h, before the addition of AngII.

### Measurement of the surface area of cardiomyocytes

After being digested, centrifuged and resuspended in DMEM, the number of cardiomyocytes was counted for at least three dishes in each group using phase-contrast microscopy. The cellular surface area was measured by the Daheng Image Analysis System. Ten fields were randomly

chosen for each group and 10 cardiomyocytes determined for each field.

#### *RNA extraction and reverse transcription-polymerase chain reaction (RT-PCR)*

Total RNA from neonatal rat cardiomyocytes was extracted according to the Trizol Reagent protocol, re-dissolved in 20  $\mu$ L of diethylpyrocarbonate (DEPC)-treated water, and stored at  $-70^{\circ}\text{C}$ . RNA was quantified by spectrophotometric means by measuring the optical density of samples at 260 nm and 280 nm. The nucleotide sequence of the primers used was: (1) ANP: sense 5-ggc tcc ttc tcc atg acc aa-3, antisense 5-tgt tat ctt cgg tac cg-3; (2)  $\beta$ -actin: sense 5-gaa gtg tga cgt tga cat ccg-3, antisense 5-tgc tga tcc aca tct gct gga-3. This yielded predicted products of 458 bp and 250 bp, respectively. RT-PCR was carried out according to the manufacturer's protocol. The cycling conditions were: one cycle of  $94^{\circ}\text{C}$  for 2 min; 35 cycles of  $94^{\circ}\text{C}$  for 30 s,  $58^{\circ}\text{C}$  for 30 s and  $72^{\circ}\text{C}$  for 1 min; and  $72^{\circ}\text{C}$  for 8 min. The products underwent electrophoresis on a 1.2% agarose gel and were visualized using ethidium bromide. The optical density of each band was measured using a BioRad Chemi DocTM EQ densitometer and BioRad Quantity One software (BioRad Laboratories, Hercules, CA, USA).  $\beta$ -actin was used as an internal control for the semi-quantitative assay.

#### *Immunostaining of phalloidin*

After the treatment as described above, neonatal rat cardiomyocytes were washed phosphate-buffered saline (PBS). They were then fixed with 4% formaldehyde in PBS for 10 min, permeabilized with 0.3% Triton X-100 in PBS for 10 min, and blocked in 1% bovine serum albumin (BSA) plus 0.1% Tween 20 for 30 min. Cardiomyocytes were stained with FITC-conjugated phalloidin for 2 h, washed in PBS and 0.1% Tween 20, and mounted for imaging using a fluorescence microscope (Leica Corporation, Wetzlar, Germany) as previously described [12].

#### *Measurement of levels of polyamines and NO*

Polyamine concentrations in cardiomyocytes were measured based on the previously described reversed-phase high-performance liquid chromatography (RP-HPLC) method [13]. Briefly, extracts were mixed with 10 nmol/L internal standard (1,6-hexanediamine), treated with benzoyl chloride and extracted with chloroform. The derivatives were separated on a Waters ODS C18 column (250 mm  $\times$  4.6 mm  $\times$  5 mm; Waters Corporation, USA) and eluted with methanol and distilled water (65:35 V:V) at  $40^{\circ}\text{C}$ . Elution was monitored by a ultraviolet detector at 229 nm (SPD-66A, Shimadzu, Japan). Polyamines were measured by HPLC (LC-6A, Shimadzu, Japan) and polyamine concentration expressed as nmol/mg of protein.

NO content in the culture medium were determined via colorimetric means with the NO detection kit (Nanjing Jiancheng Bioengineering Institute). The concentration of the resultant chromophore was determined at 550 nm using a spectrophotometer (US-640 UV; Beckman, Fullerton, CA, USA).

#### *Western blot analyses*

Total proteins of the neonatal rat cardiomyocytes were prepared according to manufacturer's instructions [14, 15]. The medium was removed at the end of the incubation. Cells were washed twice with ice-cold PBS and incubated in cool protein lysate containing the protease inhibitor phenylmethylsulphonylfluoride (PMSF) for 15 min. They were centrifuged at  $1400 \times g$  for 15 min at  $4^{\circ}\text{C}$  to remove nuclei and undisturbed cells. The protein concentration in the supernatant was determined using the Bradford protein assay with BSA as the standard. Protein samples (40  $\mu$ g) from experimental groups were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose membranes by electroblotting (300 mA for 2 h). Membranes were blocked in TBS-T [137 mM NaCl, 20 mM Tris (pH 7.6), and 0.1% (v/140v) Tween 20] containing 5% (w/v) skimmed milk at  $37^{\circ}\text{C}$  for 1 h. Membranes were then incubated overnight at  $4^{\circ}\text{C}$  with antibody against eNOS (1:500), anti-PKG-I (1:1500), c-Fos (1:500) and c-Myc (1:500). Membranes were then incubated with secondary antibody AP-IgG (Promega) diluted 1:5000 in TBS-T for 1 h at room temperature. The volumes of the protein bands were quantified by a BioRad Chemi DocTM EQ densitometer and BioRad Quantity One software (BioRad laboratories, Hercules, USA).  $\beta$ -actin was used as an internal control for the semi-quantitative assay.

#### *Immunostaining of c-Fos and c-Myc*

Isolated neonatal ventricular myocytes were placed onto coverslips, which were covered with polylysine in 24-hole culture plates. After 72 h at  $37^{\circ}\text{C}$ , cells were incubated with 10  $\mu$ g/mL of polyclonal antibody against c-Fos and c-Myc overnight at  $4^{\circ}\text{C}$ . Cells were then incubated with secondary IgG (1:50) conjugated with FITC for 15 min at  $37^{\circ}\text{C}$ . Fluorescence images were collected with a fluorescence microscope (Leica). As a control, some coverslips were incubated without primary antibody.

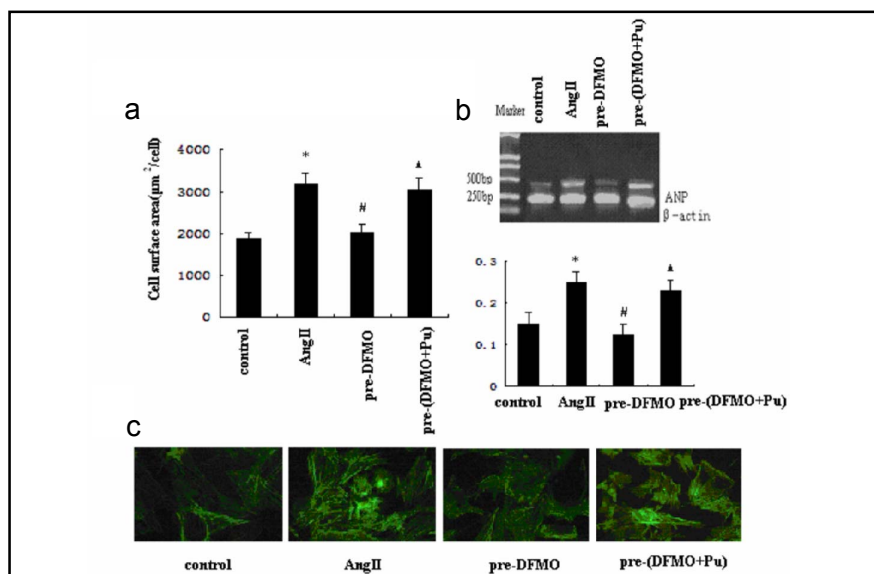
#### *Fluo-3/acetoxymethyl (Fluo-3/AM) measurements of $[\text{Ca}^{2+}]_i$*

After the treatment described above, neonatal rat cardiomyocytes were incubated with 5  $\mu$ mol/L Fluo-3/AM for 30 min at  $37^{\circ}\text{C}$ . Cardiomyocytes were rinsed twice with  $\text{Ca}^{2+}$ -free PBS to remove the remaining dye, and further incubated in DMEM. Changes in the intracellular concentration of calcium ions ( $[\text{Ca}^{2+}]_i$ ) were represented as fluorescence intensity (FI). During the experiment, the FI of Fluo-3 in cardiomyocytes was recorded for 5 min using confocal laser scanning microscope (Olympus, Tokyo, Japan) with excitation at 488 nm and emission at 530 nm. The FI was observed in eight randomly chosen cells to calculate the mean FI for all cardiomyocytes.

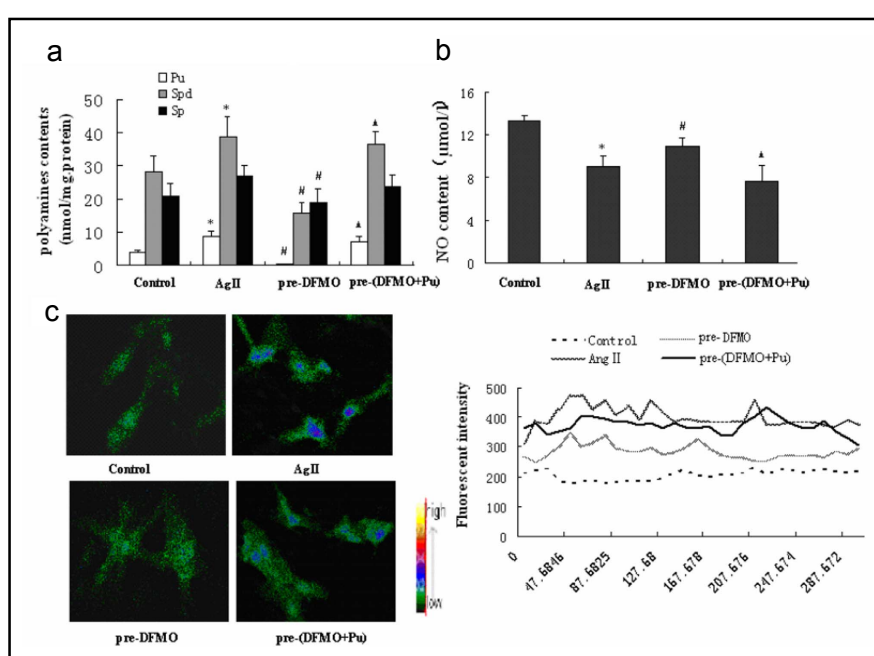
#### *Statistical analyses*

Data were obtained from at least three independent experiments that were repeated 2-4 times for each condition. Values are means  $\pm$  SEM. Comparisons among the groups were carried out using Kruskal-Wallis one-way ANOVA.  $P < 0.05$  was considered significant.

**Fig. 1.** Hypertrophic status of neonatal rat cardiomyocytes. (a) The surface area of neonatal rat cardiomyocytes. (b) mRNA expression of ANP determined by RT-PCR in neonatal rat cardiomyocytes. Levels of ANP mRNA were quantified by the densitometric analyses. Data are means $\pm$ SEM of six separate experiments. \* $P$ <0.05 versus control group, # $P$ <0.05 versus AngII group,  $\blacktriangle$   $P$ <0.05 versus pre-DFMO group. (c) Sarcomere organization in neonatal rat cardiomyocytes. Cardiomyocytes stained with FITC-conjugated phalloidin. DFMO decreased the sarcomere organization induced by AngII, and addition of putrescine reversed the effect of DFMO.



**Fig. 2.** (a) High-performance liquid chromatography (HPLC) analyses of polyamine contents in neonatal rat cardiomyocytes. (b) NO contents in neonatal rat cardiomyocytes in different groups. Data means $\pm$ SEM of four separate experiments. \* $P$ <0.05 versus control group, # $P$ <0.05 versus AngII group,  $\blacktriangle$   $P$ <0.05 versus pre-DFMO group. (c) Changes in intracellular calcium concentration ( $[\text{Ca}^{2+}]_i$ ) in neonatal rat cardiomyocytes. Fluorescence intensities in  $[\text{Ca}^{2+}]_i$  were recorded by confocal laser scanning microscopy in different treatments. AngII significantly increased  $[\text{Ca}^{2+}]_i$  ( $P$ <0.05 versus control), and this increase was attenuated by DFMO; this attenuation was reversed by the addition of putrescine ( $P$ <0.05 versus pre-DFMO).



## Results

### Hypertrophic status of neonatal rat cardiomyocytes

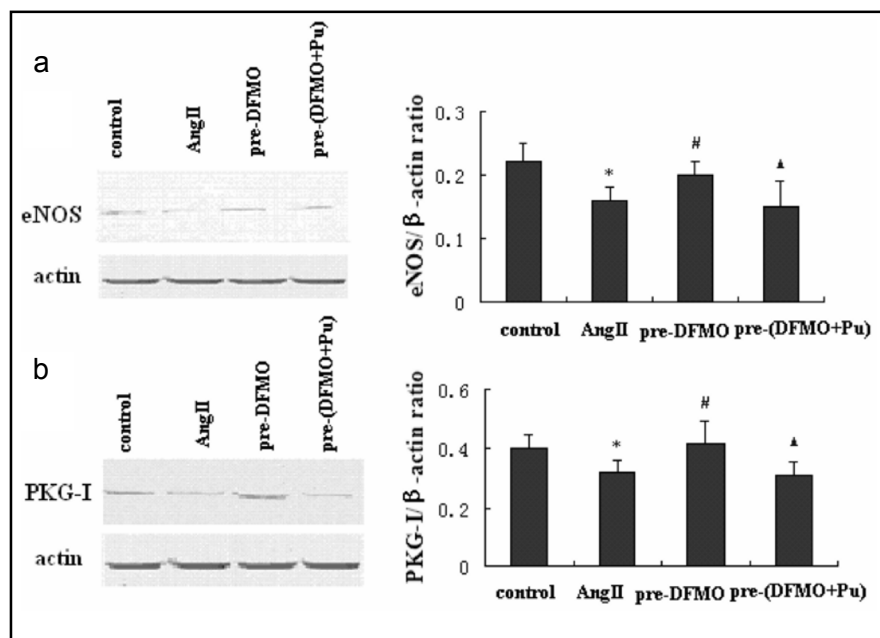
Cardiac hypertrophy is characterized by increased cardiomyocyte area and induction of fetal gene expression. To evaluate the extent of cardiac hypertrophy, the surface area of cardiomyocytes, mRNA expression of ANP and sarcomere organization were determined.

AngII increased the surface area of cardiomyocytes and upregulated mRNA expression of ANP ( $P$ <0.05 versus control group). Pretreatment with DFMO (an inhibitor of ODC that is involved in polyamine biosynthesis)

significantly reduced these AngII-induced changes ( $P$ <0.05 versus AngII group). These changes induced by AngII were similar to those observed when 500  $\mu\text{M}$  putrescine was added to cardiomyocytes together with DFMO ( $P$ <0.05 versus DFMO group) (Fig. 1a and b).

It has also been shown that organization of actin fibers into myofibrils is a major characteristic of cardiac hypertrophy. Sarcomere organization was determined using staining with FITC-conjugated phalloidin. Phalloidin staining in the control group was minimal (Fig. 1c) but became more prominent and thicker after stimulation with AngII for 48 h (Fig. 1c), indicating that sarcomere organization increased. DFMO reduced sarcomere

**Fig. 3.** (a) Protein expression of eNOS as determined by Western blot analyses in neonatal rat cardiomyocytes. (b) Protein expression of PKG-I determined by Western blot analyses in neonatal rat cardiomyocytes. Left panel represents western blots showing protein levels of eNOS and PKG-I. The right panel shows the relative immunoreactivity ratio with  $\beta$ -actin. Compared with the control group, AngII reduced the expression of eNOS and PKG-I, whereas the presence of DFMO inhibited the decrease in AngII-mediated eNOS and PKG-I expressions. Data are means  $\pm$  SEM of four separate experiments. \* $P < 0.05$  versus control group, # $P < 0.05$  versus AngII group,  $\blacktriangle P < 0.05$  versus the pre-DFMO group.



organization (Fig. 1c); a similar trend was observed between the AngII group and the pre-(DFMO+Pu) group (Fig. 1c).

#### *Levels of polyamine and NO*

We determined changes in the content of polyamines and NO in cardiomyocytes. The concentration of polyamines and NO were measured by HPLC and spectrophotometry, respectively. AngII increased the level of putrescine and spermidine, and reduced the level of NO ( $P < 0.05$  versus control group) (Fig. 2a and b). Pretreatment with DFMO reduced polyamine content and increased NO content ( $P < 0.05$  versus AngII group), but these changes were completely reversed by addition of exogenous putrescine ( $P < 0.05$  versus pre-DFMO group).

#### *Measurement of $[Ca^{2+}]_i$*

We investigated whether downregulation of the ODC/polyamine system could induce changes in  $[Ca^{2+}]_i$  in AngII-treated cardiomyocytes. AngII significantly increased  $[Ca^{2+}]_i$  ( $P < 0.05$  versus control group), and this increase in  $[Ca^{2+}]_i$  was reduced by polyamine depletion ( $P < 0.05$  versus AngII group) (Fig. 2c).  $[Ca^{2+}]_i$  was increased by addition of exogenous putrescine ( $P < 0.05$  versus pre-DFMO group).

#### *Expression of eNOS and PKG-I*

We analyzed the protein expression of eNOS and PKG-I using western blotting in neonatal rat cardiomyocytes. AngII decreased the expression of

eNOS and PKG-I ( $P < 0.05$  versus control group). Downregulation of polyamine levels significantly increased the expression of eNOS and PKG-I ( $P < 0.05$  versus AngII group). These changes were completely reversed by the addition of exogenous putrescine ( $P < 0.05$  versus pre-DFMO group).

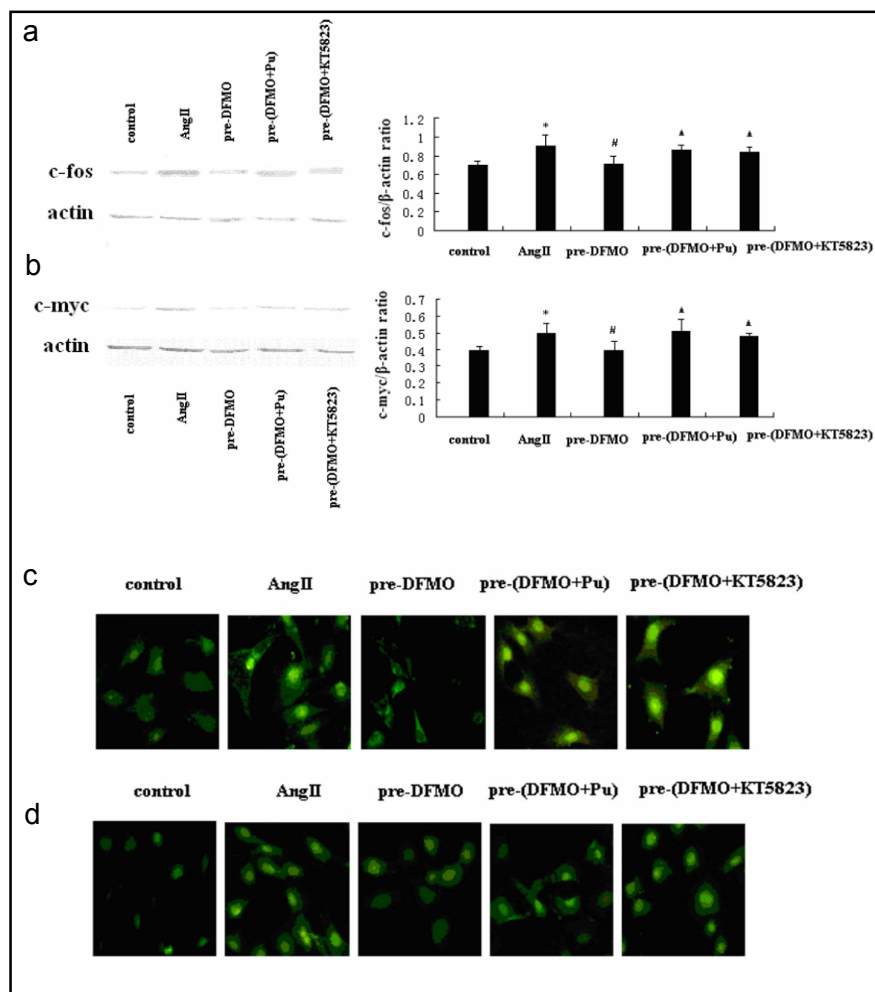
#### *Immunofluorescence of c-Fos and c-Myc*

In health, the proto-oncogene c-fos is expressed at a minimal level in the mammalian heart. Increased expression of proto-oncogenes has been implicated in the development of cardiac hypertrophy [16]. To discover if downregulation of polyamine contents modulates AngII-induced expression of c-Fos and c-Myc through the PKG pathway in cardiomyocytes, protein expressions of c-Fos and c-Myc were examined by western blotting and immunofluorescence. AngII increased the expressions of c-Fos and c-Myc, and increased nuclear translocation ( $P < 0.05$  versus control group), and these increases in cardiomyocytes treated with AngII were significantly reduced by DFMO pretreatment ( $P < 0.05$  versus AngII group) (Fig. 4a and b). Putrescine and KT5823 (specific inhibitor of PKG-I) clearly upregulated expressions of c-Fos and c-Myc and increased nuclear translocation ( $P < 0.05$  versus pre-DFMO group).

## **Discussion**

Polyamines (spermine, spermidine and putrescine) are intrinsic constituents of all eukaryotic cells. They have

**Fig. 4.** Protein expression of c-fos and c-myc determined by Western blot analyses in neonatal rat cardiomyocytes is shown in (a) and (b). Left panel represents western blots showing protein levels of c-fos and c-myc. The right panel shows the relative immunoreactivity ratio with  $\beta$ -actin. Compared with the control group, AngII increased the expression of c-fos and c-myc, whereas the presence of DFMO inhibited the increase in AngII-mediated c-fos and c-myc expression; these attenuations were reversed by the addition of putrescine or KT5823. Data are means  $\pm$  SEM of four separate experiments. \* $P < 0.05$  versus control group, # $P < 0.05$  versus AngII group,  $\blacktriangle P < 0.05$  versus the pre-DFMO group. Protein expression of c-fos and c-myc determined by immunofluorescence in neonatal rat cardiomyocytes is shown in (c) and (d). Compared with the control group, AngII increased the expression of c-fos and c-myc, whereas the presence of DFMO inhibited the increase in AngII-mediated c-fos and c-myc expression and nuclear translocation; these attenuations were reversed by the addition of putrescine or KT5823.



essential roles in the growth and differentiation of cells, the transition of membrane permeability in mitochondria, and cytosolic  $\text{Ca}^{2+}$  homeostasis [7]. Several studies suggest that the growth responses of cardiac tissue are accompanied by an increase in polyamine synthesis. Other investigations confirm a prominent role for polyamines in compensatory cardiovascular hypertrophy associated with hypertension [17-19]. The importance of polyamines in cell growth is well known, but their exact role in cardiac hypertrophy at the molecular level is incompletely understood.

In the present study, we demonstrated that downregulation of the ODC/polyamine system can attenuate AngII-induced cardiac hypertrophy, and that the anti-hypertrophy effect is mediated by PKC-I, which may be involved in NO regulation. In agreement with the findings of the present study, this NO/cGMP signaling pathway has been reported to play an important part in regulating the contractile properties of cardiac muscle *in vitro* and *in vivo* [20].

Cardiac hypertrophy is characterized by activation of the fetal gene program (e.g., ANP), an increase in cell surface area and protein synthesis [21, 22]. Organization of actin fibers into myofibrils is also a major characteristic of cardiac hypertrophy, a growth response observed in terminally differentiated cardiomyocytes [23]. Our previous results showed that isoproterenol significantly induced cardiac hypertrophy, which was related to the enhancement of polyamine metabolism [9]. Several recent studies have demonstrated that downregulation of polyamines pools by DFMO (a specific and irreversible inhibitor of ODC) attenuates growth responses in several tissues [24, 25]. In this experiment, we observed that AngII increased the surface area of cardiomyocytes, up-regulated the mRNA expression of ANP, promoted sarcomere organization, and increased the total polyamine pool levels of putrescine and spermidine. Pretreatment with DFMO can attenuate the parameters of cardiac hypertrophy and downregulate total polyamine pool levels. Our findings suggest that upregulation of the polyamine

system is an important step in cardiac hypertrophy, and that downregulation of polyamine pool levels can inhibit cardiac hypertrophy.

Polyamines share a common precursor with NO. Accumulating evidence shows that the polyamine and NO pathways are inter-regulated [10, 11]. However, whether the anti-hypertrophic role of polyamine depletion is mediated by NO and PKG-I is not known. We have showed that AngII induced cardiac hypertrophy accompanied by upregulation of polyamines and downregulation of NO, therefore, we hypothesize that downregulation of polyamine levels could inhibit AngII-induced cardiac hypertrophy involving an increased level of cellular NO; this causes activation of the PKG pathway and consequent decrease in  $[Ca^{2+}]_i$  and expression of c-Fos and c-Myc.

Extensive evidence indicates that NO is the endogenous ligand for soluble guanylate cyclase. PKG-I has been identified as the prime downstream target mediating the anti-hypertrophic effects of NO and cGMP [19, 20]. We found that DFMO pretreatment inhibited cardiac hypertrophy and upregulated the expressions of PKG-I and eNOS, and that the presence of exogenous putrescine significantly inhibited the DFMO-induced upregulation of PKG-I and eNOS. These findings suggest that downregulation of polyamine levels could activate the PKG-I signaling pathway and eventually inhibit cardiac hypertrophy. It is likely that AngII induces the ODC/polyamine system and downregulates the NO/PKG-I pathway in sequence.

$[Ca^{2+}]_i$  is a major focus in the field of cellular cardiology. Fiedler et al. reported that the growth-inhibitory effects of the NO-cGMP-PKG-I pathway upstream from calcineurin are mediated by inhibition of the L-type  $Ca^{2+}$  channel current in cardiomyocytes [26]. cGMP/PKG-I-dependent inhibition of the L-type  $Ca^{2+}$  channel current has been implicated in the negative inotropic effects of NO in cardiomyocytes [27, 28]. We observed that treatment with DFMO significantly inhibited the AngII-induced upregulation of release of  $[Ca^{2+}]_i$ .

To confirm our hypothesis, the role of KT5823 (specific inhibitor of PKG-I) on c-Fos and c-Myc in

cardiac hypertrophy was observed. In health, the proto-oncogene c-Fos is expressed at a minimal level in the mammalian heart. Increased expression of proto-oncogenes has been implicated in the development of cardiac hypertrophy. Induction of c-Fos, as a transcriptional factor, interacts with cis-regulatory elements (e.g., activator protein 1) in many genes [29]. We found that DFMO downregulated the expression of c-Fos and c-Myc induced by AngII, and that the effect was reversed by pretreatment with KT5823 or putrescine. These findings indicate that polyamine depletion could downregulate expression of transcriptional factors in the NO/PKG-I-dependent pathway.

These findings suggest that inhibition of cardiomyocyte hypertrophy is dependent (at least in part) on downregulation of polyamine synthesis, and that the decrease in polyamine levels may exert protective effects by activating the NO/PKG-I-dependent pathway and decrease in  $[Ca^{2+}]_i$ . This is the first study to assess the effect of polyamine and NO on cardiomyocyte hypertrophy, and the first observation of the interaction between the polyamine system and PKG-I signaling in rat hearts.

The findings of the present study provide the molecular mechanisms of downregulation of polyamines as negative regulators in the AngII-induced hypertrophic response of cardiomyocytes. These data may allow the development of new strategies for the treatment of cardiac hypertrophy and heart failure.

## Acknowledgements

This work was supported by the National Basic Research Program of China (973 Programme, NO.2007CB512000), the National Natural Science Foundation of China (NO.30811120280, 30470688, 30700288, 30871012), Youth Science and Technology Special Foundation in Heilongjiang Province (QC07C109) and Young academic backbone support program of ordinary high school in Heilongjiang Province(1153G055).

## References

- Mann DL: Mechanisms and models in heart failure: A combinatorial approach. *Circulation* 1999;100:999-1008.
- Hunter JJ, Chien KR: Signaling pathways for cardiac hypertrophy and failure. *N Engl J Med* 1999;341:1276-1283.
- Molkentin JD, Dorn GW 2nd: Cytoplasmic signaling pathways that regulate cardiac hypertrophy. *Annu Rev Physiol* 2001;63:391-426.



- 4 Frey N, McKinsey TA, Olson EN: Decoding calcium signals involved in cardiac growth and function. *Nat Med* 2000;6:1221-1227.
- 5 Bueno OF, Molkentin JD: Involvement of extracellular signal-regulated kinases 1/2 in cardiac hypertrophy and cell death. *Circ Res* 2002;91:776-781.
- 6 Chen QM, Tu VC, Purdon S, Wood J, Dilley T: Molecular mechanisms of cardiac hypertrophy induced by toxicants. *Cardiovasc Toxicol* 2001;1:267-283.
- 7 Ignarro LJ, Buga GM, Wei LH, Bauer PM, Wu G, del Soldato P: Role of the arginine-nitric oxide pathway in the regulation of vascular smooth muscle cell proliferation. *Proc Natl Acad Sci USA* 2001;98:74202-4208.
- 8 Salvi M, Toninello A: Effects of polyamines on mitochondrial Ca<sup>2+</sup> transport. *Biochim Biophys Acta* 2004;1661:113-124.
- 9 Lin Y, Wang LN, Xi YH, Li HZ, Xiao FG, Zhao YJ, Tian Y, Yang BF, Xu CQ: L-arginine inhibits isoproterenol-induced cardiac hypertrophy through nitric oxide and polyamine pathways. *Basic Clin Pharmacol Toxicol* 2008; 103:124-130.
- 10 Wallace JL, Ignarro LJ, Fiorucci S: Potential cardioprotective actions of no-releasing aspirin. *Nat Rev Drug Discov* 2002;5:375-382.
- 11 Mühling J, Engel J, Halabi M, Müller M, Fuchs M, Krüll M, Harbach H, Langefeld TW, Wolff M, Matejec R, Welters ID, Menges T, Hempelmann G: Nitric oxide and polyamine pathways-dependent modulation of neutrophil free amino- and  $\alpha$ -keto acid profiles or host defense capability. *Amino Acids* 2006; 31:11-26.
- 12 Thorburn J, Frost JA, Thorburn A: Mitogen-activated protein kinases mediate changes in gene expression, but not cytoskeletal organization associated with cardiac muscle cell hypertrophy. *J Cell Biol* 1994;126:1565-1572.
- 13 Manni A, Washington S, Griffith JW, Verderame MF, Mauger D, Demers LM, Samant RS, Welch DR: Influence of polyamines on in vitro and in vivo features of aggressive+and metastatic behavior by human breast cancer cells. *Clin Exp Metastasis* 2002;19:95-105.
- 14 Maiti S, Chen X, Chen G. All-trans retinoic acid induction of sulfotransferases. *Basic Clin Pharmacol Toxicol* 2005;96:44-53.
- 15 Xu C, Lu Y, Tang G, Wang R: Expression of voltage-dependent K(+) channel genes in mesenteric artery smooth muscle cells. *Am J Physiol* 1999; 277:G1055-1063.
- 16 Cheng TH, Shih NL, Chen SY, Lin JW, Chen YL, Chen CH, Lin H, Cheng CF, Chiu WT, Wang DL, Chen JJ: Nitric oxide inhibits endothelin-1-induced cardiomyocyte hypertrophy through cGMP-mediated suppression of extracellular-signal regulated kinase phosphorylation. *Mol Pharmacol* 2005;68:1183-1192.
- 17 Ibrahim J, Schachter M, Hughes AD, Sever PS: Role of polyamines in hypertension induced by angiotensin II. *Cardiovasc Res* 1995;29:50-56.
- 18 Lipke DW, Newman PS, Tofiq S, Guo H, Arcot SS, Aziz SM, Olson JW, Soltis EE: Multiple polyamine regulatory pathways control compensatory cardiovascular hypertrophy in coarctation hypertension. *Clin Exp Hypertens* 1997;19:269-295.
- 19 Shantz LM, Feith DJ, Pegg AE: Targeted overexpression of ornithine decarboxylase enhances beta-adrenergic agonist-induced cardiac hypertrophy. *Biochem J* 2001;358:25-32.
- 20 Paulus WJ, Bronzwaer JG: Nitric oxide's role in the heart: control of beating or breathing? *Am J Physiol* 2004;287:H8-H13.
- 21 Komuro I, Yazaki Y: Control of cardiac gene expression by mechanical stress. *Annu Rev Physiol* 1993;55:55-75.
- 22 Sadoshima J, Izumo S: The cellular and molecular response of cardiac myocytes to mechanical stress. *Annu Rev Physiol* 1997;59:551-571.
- 23 Sadoshima J, Izumo S: Molecular characterization of angiotensinII-induced hypertrophy of cardiac myocytes and hyperplasia of cardiac fibroblasts: critical role of the AT1 receptor subtype. *Circ Res* 1993;73:413-423.
- 24 Pegg AE: Polyamine metabolism and its importance in neoplastic growth and as a target for chemotherapy. *Cancer Res* 1988; 48:759-774.
- 25 Ray RM, Zimmerman BJ, McCormack SA, Patel TB, Johnson LR: Polyamine depletion arrests cell cycle and induces inhibitors p21(Waf1/Cip1), p27(Kip1), and p53 in IEC-6 cells. *Am J Physiol* 1999;276:C684-691.
- 26 Fiedler B, Lohmann SM, Smolenski A, Linnemuller S, Pieske B, Schroder F, Molkentin JD, Drexler H, Wollert KC: Inhibition of calcineurin-NFAT hypertrophy signaling by cGMP-dependent protein kinase type I in cardiac myocytes. *Proc Natl Acad Sci U S A* 2002;99:11363-11368.
- 27 Wegener JW, Nawrath H, Wolfgruber W, Kühbandner S, Werner C, Hofmann F, Feil R: cGMP-dependent protein kinase I mediates the negative inotropic effect of cGMP in the murine myocardium. *Circ Res* 2002;90:18-20.
- 28 Schröder F, Klein G, Fiedler B, Bastein M, Schnasse N, Hillmer A, Ames S, Gambaryan S, Drexler H, Walter U, Lohmann SM, Wollert KC: Single L-type Ca<sup>2+</sup> channel regulation by cGMP-dependent protein kinase type I in adult cardiomyocytes from PKG I transgenic mice. *Cardiovasc Res* 2003;60:268-277.
- 29 Parameswaran N, Hall CS, McCabe LR, Spielman WS: Adrenomedullin increases AP-1 expression in rat mesangial cells via activation of protein kinase-A and p38 MAPK. *Cell Physiol Biochem* 2003;13:367-374.