Journal of Zhejiang University SCIENCE B
ISSN 1673-1581 (Print); ISSN 1862-1783 (Online)
www.zju.edu.cn/jzus; www.springerlink.com
E-mail: jzus@zju.edu.cn

Effects of combination of irbesartan and perindopril on calcineurin expression and sarcoplasmic reticulum Ca^{2+}-ATPase activity in rat cardiac pressure-overload hypertrophy

JIANG Qing-jun[1,2], XU Geng[†‡1], MAO Fei-fei[3], ZHU You-fa[4]

([1]*Department of Cardiology, the Second Affiliated Hospital, School of Medicine, Zhejiang University, Hangzhou 310009, China*)

([2]*Department of Cardiology, Ningbo Lihuili Hospital, Ningbo 315041, China*)

([3]*Department of Pharmacy, Children's Hospital, School of Medicine, Zhejiang University, Hangzhou 310029, China*)

([4]*Department of Pathology, School of Medicine, Zhejiang University, Hangzhou 310009, China*)

[†]E-mail: xugeng@21cn.com

Received June 24, 2005; revision accepted Nov. 13, 2005

Abstract: Aim: To observe effects of angiotensin (Ang) II receptor antagonist (AT1) irbesartan and angiotensin-converting enzyme (ACE) inhibitor perindopril on rat myocardium calcineurin expression and sarcoplasmic reticulum Ca^{2+}-ATPase activity in the model of pressure-overload cardiac hypertrophy. Methods: Forty male adult Sprague Dawley rats were divided into 5 groups. One group was treated by sham operation; four groups were myocardium hypertrophy cases caused by banding aortic above renal artery. Drugs were given one week after operation. Group 1: sham group, rats (n=8) were gavaged with normal saline 2 ml/(kg·d) (ig); Group 2: control group, rats (n=8) were treated with normal saline 2 ml/(kg·d) (ig); Group 3: rats (n=8) were given perindopril 2 mg/(kg·d) (ig); Group 4: rats (n=8) were treated with irbesartan 20 mg/(kg·d) (ig); Group 5: rats (n=8) were given irbesartan 20 mg/(kg·d) plus perindopril 2 mg/(kg·d) (ig). Morphometric determination, calcineurin expression and sarcoplasmic reticulum Ca^{2+}-ATPase activity were done at the end of 6 week of drug intervention. Expression of calcineurin in myocardium was detected by immunohistochemistry. Results: Left ventricular mass index (LVMI), transverse diameter of myocardial cell (TDM), calcineurin activity were remarkably decreased after drug intervention and this decrease was most remarkable in the combination drug therapy group. Sarcoplasmic reticulum Ca^{2+}-ATPase activity was increased after drug intervention, especially in the combined drug therapy group. Calcineurin expression in myocardium was remarkably decreased after drug intervention. LVMI was positively correlated with TDM and calcineurin, negatively correlated with sarcoplasmic reticulum Ca^{2+}-ATPase. Conclusion: These data suggest that irbesartan and perindopril inhibit cardiac hypertrophy through the increased activity of sarcoplasmic reticulum Ca^{2+}-ATPase and decreased expression of calcineurin. Their combination had better effects on regressing of ventricular hypertrophy.

Key words: Angiotensin (Ang) II receptor antagonist, Angiotensin-converting enzyme inhibitor, Calcineurin, Sarcoplasmic reticulum Ca^{2+}-ATPase, Pressure overload, Cardiac hypertrophy, Rat

doi:10.1631/jzus.2006.B0228 **Document code:** A **CLC number:** R363

INTRODUCTION

Left ventricular hypertrophy has been thought to be the principal predicators of predisposing risk factor of cardiac morbidity and mortality (Devereux, 1995; Levy *et al*., 1990). The pathogenesis that mediates cardiac hypertrophy is poorly understood. Cardiac hypertrophy can be induced by hemodynamic overload, ischemic disease, neurohumoral factors and intrinsic defects in cardiac structural protein genes (Sadoshima and Izumo, 1997; Vikstrom and Leinwand, 1996). Another intracellular regulatory pathway implicated in cardiac hypertrophy involves the calcium and calmodulin dependent protein phosphatase calcineurin and the transcription factor NF-AT3 (Molkentin, *et al*., 1998). Cardiac overex-

‡ Corresponding author

pression by the transgene of either activated calcineurin or a constitutively nuclear NF-AT3 mutant produces substantial hypertrophy that rapidly progresses to heart failure. These studies are extended to demonstrate prevention of phenotypic hypertrophy with cyclosporine or FK506 in genetically altered mouse models of cardiomyopathy and in a pathophysiological model of pressure-overload hypertrophy in the rat (Sussman *et al*., 1998; Shimoyama *et al*., 1999). In contrast, recent studies reported that the calcineurin is not involved in the development of cardiac hypertrophy (Zhang *et al*., 2000). In the present study we further investigated (1) the relationship between the expression of myocardial calcineurin and cardiac hypertrophy; (2) the effect of angiotensin (Ang) II receptor antagonist (AT1) irbesartan and angiotensin-converting enzyme (ACE) inhibitor perindopril on cardiac hypertrophy by measuring left ventricular mass index (LVMI), transverse diameter of myocardial cell (TDM), expression and activity of calcineurin and sarcoplasmic reticulum Ca^{2+}-ATPase in the rat myocardium with rat model of pressure-overloaded cardiac hypertrophy.

MATERIALS AND METHODS

Experimental design

Forty male adult Sprague Dawley rats (Grade SPF, Certificate No. 003, male, weighing 160~180 g) were purchased from the Shanghai Laboratory Animal Center and divided into 5 groups. One group was treated by sham operation, four groups were myocardium hypertrophy cases caused by banding aortic (Doering *et al*., 1988): the abdominal aorta above renal artery was constricted to a diameter of 0.6 mm, equivalent to the external diameter of a #5 needle, which was included in and then withdrawn from a ligature. In the sham group, the same surgical operations were performed as in the other four groups except that the aorta was constricted. Group 1: sham group, rats (n=8) were gavaged with normal saline 2 ml/(kg·d) (ig); Group 2: control group, rats (n=8) were treated with normal saline 2 ml/(kg·d) (ig); Group 3: rats (n=8) were administered perindopril 2 mg/(kg·d) (ig); Group 4: rats (n=8) received irbesartan 20 mg/(kg·d) (ig); Group 5: rats (n=8) were given irbesartan 20 mg/(kg·d) (ig) plus perindopril 2 mg/(kg·d) (ig). Perindopril and irbesartan was donated by Servier Industries (France) and Sanofi Winthrop Industries (France), respectively.

Body weight was recorded daily. Eight rats of each group were studied 6 weeks following one week aortic constriction. At the end of the 6-week drug intervention, the rats were anesthetized by pentobarbital (30 mg/kg, i.p). After body weighing, the heart was rapidly removed and perfused with normal saline. Moisture content of the heart was absorbed with filter paper. The weight of the left ventricle and ventricular septum served as left ventricular mass (LVM). Ratio of LVM and body weight (BW) was calculated as the index of left ventricular hypertrophy. After the left ventricle and ventricular septum were weighed, the myocardial tissue was either snap-frozen in liquid nitrogen for observing the activity of calcineurin and sarcoplasmic reticulum Ca^{2+}-ATPase, or fixed in buffered formalin for histopathology. The left ventricular myocardium was processed and embedded in paraffin for transverse sectioning (4 μm) and stained with haematoxylin/eosin (HE). The transverse diameter of myocardial cell (TDM) was examined with optic microscope. Twenty myocardial cells were randomly examined in each slice and average values of TDM were calculated.

Calcineurin phosphatase assay

Calcineurin activity was measured with the method described by Lim *et al*.(2000). The frozen rat heart was weighed and freeze-fracture pulverized to powder while frozen in liquid nitrogen. The frozen powdered tissue was homogenized at 0~4 °C in 2 volumes (V/w) of 50 mmol/L Tris (pH 7.5), 0.1 mmol/L EGTA (ethyleneglycol bis(2-aminoethyl ether) tetraacetic acid), 1 mmol/L EDTA (ethylene diamine tetraacetic acid), 0.5 mmol/L DTT (dithiothreitol), 50 mg/L PMSF (phenylmethysulfonylfluoride), 50 mg/L STI (soybean trypsin inhibitor), 5 mg/L leupeptin, 5 mg/L aprotinin, then microfuged at 12000 g for 10 min at 4 °C. Protein of supernatants was measured. Fifty μl of supernatants was added to 350 μl Substrate I (including 50 mmol/L Tris-HCl, pH 7.4, 0.5 mmol/L DTT, 0.2 g/L BSA, 10 mmol/L PNPP, 0.5 mmol/L $MnCl_2$, 0.2 mmol/L $CaCl_2$, 0.3 μmol/L calmodulin) and 350 μl Substrate II (including 50 mmol/L Tris-HCl, pH 7.4, 0.5 mmol/L DTT, 0.2 g/L BSA (bovine serum albumin), 10 mmol/L

PNPP (*p*-nitrophenylphosphate), 0.5 mmol/L $MnCl_2$, 3 mmol/L EGTA) respectively, kept at 30 °C for 10 min, then 13% K_2HPO_4 80 μl was added immediately to stop the reaction. Calcineurin activity was calculated by subtracting the activity measured in Substrate I from the activity measured in Substrate II; absorbance was determined at 410 nm. Calcineurin activity was expressed as $A_{410\,nm}$/(mg protein).

Measurement of sarcoplasmic reticulum Ca^{2+}-ATPase activity

Sarcoplasmic reticulum membranes from rat left ventricular muscle were prepared by the method described by Jones *et al*.(1979). Left ventricles were homogenized in 30 mmol/L Tris-maleate buffer containing 0.3 mol/L sucrose, 5 mg/L leupeptin and 0.1 mmol/L PMSF, at pH 7.0 (Solution 1) using a Brinkmann polytron. The homogenate was centrifuged at 5500×g for 10 min. The resultant supernatant was again filtered through four layers of cheesecloth before centrifugation at 12000×g for 20 min. The supernatant was again filtered through cheesecloth and centrifuged at 143000×g for 30 min. The pallet was suspended in a buffer of the following composition: 30 mmol/L Tris-maleate buffer containing 0.3 mol/L sucrose, 0.6 mol/L KCl, 5 mg/L leupeptin and 0.1 mmol/L PMSF, at pH 7.0 (Solution 2). This suspension was centrifuged at 143000×g for 45 min. The pellet was resuspended in Solution 2, homogenized and centrifuged at 143000×g, as described above. The pellet was suspended in Solution 1 and centrifuged at 143000×g. The final pellet was the microsomal fraction rich in sarcoplasmic reticulum vesicles, and then suspended in the following solution: 20 mmol/L Tris-maleate buffer containing 0.3 mol/L sucrose, 0.1 mol/L KCl, 5 mg/L leupeptin and 0.1 mmol/L PMSF, at pH 7.0. Ca^{2+}-ATPase activity was measured according to the direction of the kit. It was expressed as μmol Pi/(mg protein·h).

Immunohistochemistry

Formalin-fixed tissues were paraffin embedded and cut into 4-μm sections; sections which were then deparaffinized in xylene and rehydrated in graded ethanol to PBS. After blocking endogenous peroxidase activity with 3% H_2O_2/methanol (1/32, *V*/*V*) for 10 min, tissue sections were boiled for antigen epitope retrieval in 0.1 mol citrate buffer (pH 6.0) for 10 min. The tissue sections were then interacted overnight at 4 °C with the 1:200 diluted primary antibodies rabbit anti-rat calcineurin (PP2B-A) (Santa Cruz Biotechnology), controls were incubated with the normal rabbit serum and the PBS instead of primary antibodies. After thoroughly washing with PBS, the slides were incubated for 30 min with EnVision™ horseradish peroxidase-labelled secondary antibody goat anti-rabbit IgG (Dako Company) at room temperature, again washed thoroughly in PBS. The slides were then developed for 2~5 min in diaminobenzidine and rinsed with water and then counterstained with Haematoxylon. The result of immunohistochemistry was estimated by semi-quantitative scoring system of Barnes *et al*.(1993). The degree of calcineurin expression was determined by the following: (1) the intensity of staining, (2) the proportion of staining, (3) a combination of the two (Score 1×Score 2). Intensity was given scores of 0 to 3 and proportion was given scores of 0 to 4 (1% to 25%=1; 26% to 50%=2; 51% to 75%=3; and >75%=4). The slides were independently scored by two of the authors and any discrepancies were resolved by subsequent consultation.

Statistical analysis

Using the SPSS 11.0 for Windows Statistical Package, data obtained were expressed as $X\pm SD$ and analyzed by one-way ANOVA. Linear correlation analysis was used to analyze the relationship between LVMI and TDM, calcineurin, sarcoplasmic reticulum Ca^{2+}-ATPase. Statistical significance was set at $P<0.05$.

RESULTS

Histopathology and cardiac hypertrophic index

The left ventricular myocardium was stained with HE and observed under light microscope. The myocardial cell of the control group was thick hypertrophied, cardiac muscle fiber arrayed in disorder. Transverse diameter of myocardial cell (TDM) and myocardial cell nuclear of the control group were larger than those of other groups. Left ventricular mass index (LVMI) and TDM in the irbesartan group, perindopril group and combination group were remarkably decreased compared with those of the control group ($P<0.05$). LVMI in the combination group

group was remarkably decreased, compared with that in the irbesartan group or perindopril group (P<0.05) (Table 1).

Calcineurin expression in myocardium detected by immunohistochemistry

Calcineurin was mainly expressed in cytoplasma. The calcineurin expression detected by immunohistochemistry in the control group was stronger than that in any other groups (P<0.01) (Fig.1).

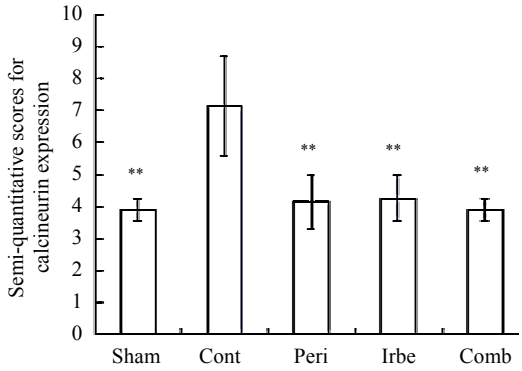

Fig.1 Expression of calcineurin in rat myocardium detected by immunohistochemistry. Semi-quantitative analysis showed that the calcineurin expression in control group was larger than that of irbesartan group, perindopril group and combination group (P<0.01)

Cont: Control; Peri: Perindopril; Irbe: Irbesartan; Comb: Combination; ** P<0.01, vs control group

Calcineurin activity

Calcineurin activities of the irbesartan group, perindopril group and combination group were remarkably decreased compared to control group (P<0.05) (Fig.2).

Sarcoplasmic reticulum Ca^{2+}-ATPase activity

Sarcoplasmic reticulum Ca^{2+}-ATPase activities of irbesartan group, perindopril group and combination group were remarkably increased compared with the control group (P<0.05). Sarcoplasmic reticulum Ca^{2+}-ATPase activity of combination group was remarkably increased compared with that of irbesartan group or perindopril group (P<0.05) (Fig.3).

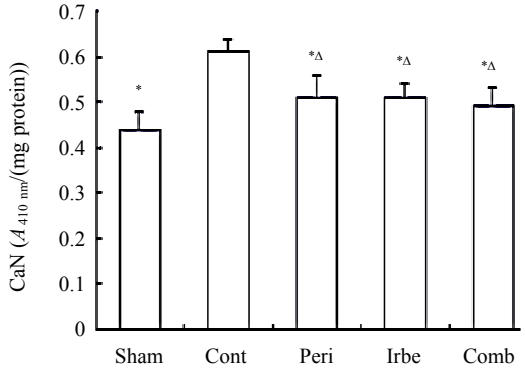

Fig.2 Calcineurin (CaN) activity of cardiac myocardium. Calcineurin activity in control group as larger than that of irbesartan group, perindopril group and combination group (P<0.05)

Cont: Control; Peri: Perindopril; Irbe: Irbesartan; Comb: Combination; * P<0.05, vs control group; Δ P<0.05, vs sham group

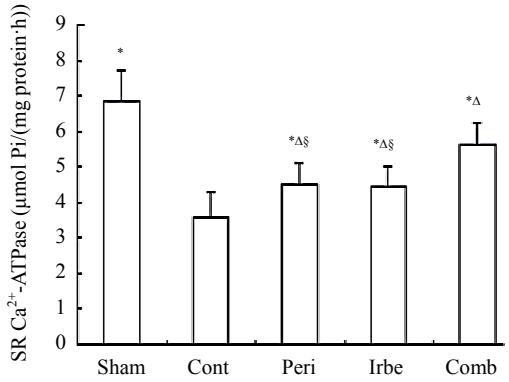

Fig.3 Sarcoplasmic reticulum (SR) Ca^{2+}-ATPase activity of rat cardiac myocardium. The sarcoplasmic reticulum Ca^{2+}-ATPase activity of normal cardiac myocardium in the sham group was very high. Sarcoplasmic reticulum Ca^{2+}-ATPase activity in the control group was lower than that of perindopril group, irbesartan group, and combination group

Cont: Control; Peri: Perindopril; Irbe: Irbesartan; Comb: Combination; * P<0.05, vs control group; Δ P<0.05, vs sham group; § P<0.05, vs combination group

Table 1 Cardiac hypertrophic index after treatment with vehicle (control), irbesartan, perindopril, and the combination of irbesartan and perindopril in rats

Group	Sham	Control	Perindopril	Irbesartan	Combination
LVMI (mg/g)	2.03±0.16*	2.99±0.16	2.39±0.16*Δ#	2.36±0.13*Δ#	2.14±0.12*
TDM (μm)	10.79±0.39*	15.13±0.35	11.73±0.37*Δ	11.59±0.42*Δ	11.43±0.33*Δ

* P<0.05, vs control group; Δ P<0.05, vs sham group; # P<0.05, vs combination group

Linear correlation analysis between LVMI and TDM, calcineurin activity, sarcoplasmic reticulum Ca^{2+}-ATPase

There was significance positive correlation between LVMI and TDM or calcineurin activity ($P<0.01$), significance negative correlation between LVMI and sarcoplasmic reticulum Ca^{2+}-ATPase ($P<0.01$) (Table 2).

Table 2 Linear correlation analysis between LVMI and TDM, calcineurin activity, sarcoplasmic reticulum Ca^{2+}-ATPase

	LVMI	
	r	*P*
TDM	0.887	<0.01
Calcineurin activity	0.800	<0.01
SR Ca^{2+}-ATPase	−0.726	<0.01

SR: Sarcoplasmic reticulum

DISCUSSION

There are different views on the combination of ACE inhibitor and AT1 receptor antagonist in the treatment of cardiovascular diseases. Val-HeFT and CHARM both showed that the combination of the two drugs was more effective in the treatment of heart failure than monotherapy with each agent. But the result of VALIANT was opposite. More studies need to be done. Till now, there are few studies on combination of the two drugs in the treatment of hypertension. In this study, we made a model of cardiac hypertrophy by the ligating aorta of rat. Seven weeks later the cardiac hypertrophy in the control group was very significant. The myocardial cell analyzed by pathology was hypertrophied, cardiac muscle fiber became thick and disorderly arrayed. LVMI, TDM of drug treatment groups were remarkably decreased compared with control group ($P<0.05$). LVMI in the combination group was remarkably decreased compared with irbesartan group or perindopril group ($P<0.05$). This indicated the combination of irbesartan and perindopril had better effects on inhibiting ventricular hypertrophy. Antihypertensive drugs irbesartan (angiotensin receptor blocker) and perindopril (angiotensin-converting enzyme inhibitor) can inhibit cardiac hypertrophy and regress ventricular remodelling (Kawano *et al*., 2000).

Cardiac left ventricular hypertrophy is an adaptive response to numerous forms of cardiovascular stimuli/stress that temporarily augments cardiac performance by reducing wall tension (Grossman *et al*., 1975). Although this response is initially beneficial, it often progresses to decompensation and heart failure if the initiating stimulus is not alleviated. Left ventricular hypertrophy is the independent risk factor of the complication of cardiovascular diseases, but its particular pathogenesis is still unknown.

Calcineurin (CaN) is a Ca^{2+} and calmodulin dependent protein phosphatase. Molkentin *et al*.(1998) found it could act as a transducer of hypertrophic signals. In vitro and in vivo studies indicated that various kinds of stimuli such as stretch, pressure overload, neuroendocrine factors (Ang II, ET-1) produce the sustained increases in cytosolic calcium which after coupling with its binding protein calmodulin, results in calcineurin activation. The activated calcineurin dephosphorylates nuclear factor of activated T-cell (NFAT). Dephosphorylated NFAT translocates to the nucleus where it interacts with another transcriptional factor (GATA4) and causes transcriptional activation of hypertrophic fetal genes leading to cardiomyocyte hypertrophy. In our study, calcineurin activity and expression of the control group was stronger than that of three drug treatment groups. Both irbesartan and perindopril decreased calcineurin expression and its activity. These results indicated that calcineurin is involved in the development of cardiac hypertrophy.

There is close relationship between cardiac hypertrophy and calcium overload of cardiac myocyte. The sarcoplasmic reticulum Ca^{2+}-ATPase is the central structure for the reuptake of Ca^{2+} in myocyte (Movsesian and Schwinger, 1998). Calcineurin significantly reduces V_{max} of sarcoplasmic reticulum Ca^{2+}-ATPase activity in human myocardium, which contributes to the resulting alteration of Ca^{2+} cycling in failing human myocardium. V_{max} of sarcoplasmic reticulum Ca^{2+}-ATPase activity is coordinately regulated by both CaM-kinase and calcineurin in human myocardium (Münch *et al*., 2002). The specific phosphatase for CaM-kinase-dependent action is the phosphatase calcineurin which is the counterpart of CaM-kinase. Calcineurin plays a prominent role in cardiac hypertrophy and failure (Molkentin *et al*., 1998; Lim and Molkentin, 1999; de Windt *et al*.,

2001). Increased protein expression of calcineurin was found in failing human myocardium (Münch *et al*., 2002). In vitro data suggested that calcineurin contributes to the reduction of phospholamban phosphorylation with subsequent impairment of the sarcoplasmic reticulum Ca^{2+}-ATPase activity in failing human myocardium. The regulation of sarcoplasmic reticulum Ca^{2+}-ATPase activity is under inhibitory control of phospholamban (James *et al*., 1989; Kimura *et al*., 1996). Phosphorylation of phospholamban increases sarcoplasmic reticulum Ca^{2+}-ATPase activity (Tada *et al*., 1975). In vitro sarcoplasmic reticulum Ca^{2+}-ATPase activity can be depressed by calcineurin-mediated dephosphorylation in non-failing tissue (Münch *et al*., 2002). When sarcoplasmic reticulum Ca^{2+}-ATPase activity is decreased, reuptake of calcium into the sarcoplasmic reticulum is decreased, which result in the Ca^{2+} overload in the myocardium cytoplasm and calcineurin as well as initiate hypertrophy in cardiomyocytes. At the early stage of cardiac hypertrophy, sarcoplasmic reticulum Ca^{2+}-ATPase activity can increase by compensation (Shen *et al*., 1991). While cardiac hypertrophy was significant, the expression of sarcoplasmic reticulum Ca^{2+}-ATPase mRNA and protein activity are remarkably reduced (Bastie *et al*., 1990). In our study sarcoplasmic reticulum Ca^{2+}-ATPase activity of the control group was reduced remarkably after treatment with irbesartan or perindopril, sarcoplasmic reticulum Ca^{2+}-ATPase activity was increased significantly, especially in the combination group whose sarcoplasmic reticulum Ca^{2+}-ATPase activity was remarkably increased compared with irbesartan group or perindopril group. Angiotensin (Ang) II receptor antagonist irbesartan and angiotensin-converting enzyme inhibitor perindopril can inhibit and regress cardiac hypertrophy by regulating the activity of sarcoplasmic reticulum Ca^{2+}-ATPase and expression of calcineurin.

CONCLUSION

These data suggest that calcineurin is involved in the development of cardiac hypertrophy. Angiotensin (Ang) II receptor antagonist irbesartan and angiotensin-converting enzyme inhibitor perindopril can inhibit cardiac hypertrophy by increasing activity of sarcoplasmic reticulum Ca^{2+}-ATPase and decreasing expression of calcineurin. Their combination has better effects in regressing ventricular hypertrophy.

References

Barnes, D.M., Dublin, E.A., Fisher, C.J., Levison, D.A., Millis, R.R., 1993. Immunohistochemical detection of p53 protein in mammary carcinoma: an important new independent indicator of prognosis? *Hum. Pathol.*, **24**(5):469-476. [doi:10.1016/0046-8177(93)90158-D]

Bastie, D., Levitsky, D., Rappaport, L., 1990. Function of the sarcoplasmic reticulum and expression of its Ca^{2+}-ATPase gene in pressure overload-induced cardiac hypertrophy in the rat. *Circ. Res.*, **66**(2):554-564.

de Windt, L.J., Lim, H.W., Bueno, O.F., Liang, Q., Delling, U., Braz, J.C., Glascock, B.J., Kimball, T.F., Monte, F.D., Hajjar, R.J., Molkentin, J.D., 2001. Targeted inhibition of calcineurin attenuates cardiac hypertrophy in vivo. *Proc. Natl. Acad. Sci. USA*, **98**:3322-3327.

Devereux, R.B., 1995. Left ventricular geometry, pathophysiology and prognosis. *J. Am. Coll. Cardiol.*, **25**(4):885-887. [doi:10.1016/0735-1097(94)00547-4]

Doering, C.W., Jalil, J.E., Janicki, J.S., 1988. Collagen network remodelling and diastolic stiffness of the rat left ventricle with pressure overload hypertrophy. *Cardiovasc. Res.*, **22**(10):686-695.

Grossman, W., Jones, D., McLaurin, L.P., 1975. Wall stress and patterns of hypertrophy in the human left ventricle. *J. Clin. Invest.*, **55**:56-64.

James, P., Inui, M., Tada, M., Chiesi, M., Carafoli, E., 1989. Nature and site of phospholamban regulation of the Ca^{2+} pump of sarcoplasmic reticulum. *Nature*, **342**(6245): 90-92. [doi:10.1038/342090a0]

Jones, L.R., Besch, H.R., Fleming, J.W., McConnaughey, M.M., Watanabe, A.M., 1979. Separation of vesicles of cardiac sarcolemma from vesicles of cardiac sarcoplasmic reticulum. Comparative biochemical analysis of component activities. *J. Biol. Chem.*, **254**(2):530-539.

Kawano, H., Do, Y.S., Kawano, Y., Stames, V., Barr, M., Law, R.E., Hsueh, W.A., 2000. Angiotensin II has multiple profibrotic effects in human cardiac fibroblasts. *Circulation*, **101**(10):1130-1137.

Kimura, Y., Kurzydlowski, K., Tada, M., MacLennan, D.H., 1996. Phospholamban regulates the Ca^{2+}-ATPase through intramembrance interaction. *J. Biol. Chem.*, **271**(42): 21726-21731. [doi:10.1074/jbc.271.42.25958]

Levy, D., Garrison, R.J., Savage, D.D., Kannel, W.B., Castelli, W.P., 1990. Prognostic implications of echocardiographically determined left ventricular mass in the Framingham heart study. *N. Engl. J. Med.*, **322**:1561-1566.

Lim, H.W., Molkentin, J.D., 1999. Calcineurin and human heart failure [letter] [see comments]. *Nat. Med.*, **5**(3):246-247. [doi:10.1038/6430]

Lim, H.W., de Windt, L.J., Steinberg, L., Taigen, T., Witt, S.A.,

Kimball, T.R., Molkentin, J.D., 2000. Calcineurin expression, activation, and function in cardiac pressure-overload hypertrophy. *Circulation*, **101**:2431-2437.

Molkentin, J.D., Lu, J.R., Antos, C.L., Markham, B., Richardson, J., Robbins, J., Grant, S.R., Olson, E.N., 1998. A calcineurin dependent transcriptional pathway for cardiac hypertrophy. *Cell*, **93**(2):215-228. [doi:10.1016/S0092-8674(00)81573-1]

Movsesian, M.A., Schwinger, R.H.G., 1998. Calcium sequestration by the sarcoplasmic reticulum in heart failure. *Cardiovasc. Res.*, **37**(2):352-359. [doi:10.1016/S0008-6363(97)00259-9]

Münch, G., Bölck, B., Karczewski, P., Schwinger, R.H.G., 2002. Evidence for calcineurin-mediated regulation of SERCA 2a activity in human myocardium. *J. Mol. Cell. Cardiol.*, **34**(3):321-334. [doi:10.1006/jmcc.2001.1515]

Sadoshima, J., Izumo, S., 1997. The cellular and molecular response of cardiac myocytes to mechanical stress. *Ann. Rev. Physiol.*, **59**(1):551-571. [doi:10.1146/annurev.physiol.59.1.551]

Shen, H., Mirsalimi, S.M., Weiler, J.E., 1991. Effects of mild cardiac hypertrophy, induced by volume overload in turkeys, on myocardial sarcoplasmic reticulum calcium-pump and calcium-channel activities and on the creatine kinase system. *Am. J. Vet. Res.*, **52**(9):1527-1530.

Shimoyama, M., Hayashi, D., Takimoto, E., Zou, Y., Oka, T., Uozumi, H., Kudoh, S., Shibasaki, F., Yazaki, Y., Nagai, R., Komuro, I., 1999. Calcineurin plays a critical role in pressure overload-induced cardiac hypertrophy. *Circulation*, **100**(24):2449-2454.

Sussman, M.A., Lim, H.W., Gude, N., Taigen, T., Olson, E.N., Robbins, J., Colbert, M.C., Gualberto, A., Wieczorek, D.F., Molkentin, J.D., 1998. Prevention of cardiac hypertrophy in mice by calcineurin inhibition. *Science*, **281**(5383):1690-1693. [doi:10.1126/science.281.5383.1690]

Tada, M., Kirchberger, M.A., Katz, A.M., 1975. Phosphorylation of a 22000-dalton component of the cardiac sarcoplasmic reticulum by adenosine 3′-5′-monophosphate-dependent protein kinase. *J. Biol. Chem.*, **250**:2640-2647.

Vikstrom, K.L., Leinwand, L.A., 1996. Contractile protein mutations and heart disease. *Curr. Opin. Cell Biol.*, **8**(1):97-105. [doi:10.1016/S0955-0674(96)80053-6]

Zhang, W., Kowal, R.C., Rusnak, F., 2000. Failure of calcineurin inhibitors to prevent pressure-overload left ventricular hypertrophy in rats. *Circ. Res.*, **84**(6):722-728.