Mechanisms of Cardiac Hypertrophy and Injury — Possible Role of Protein Kinase C Activation —

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To examine the molecular mechanisms by which mechanical stimuli induced cardiac hypertrophy and injury, we cultured rat neonatal cardiocytes in deformable dishes and imposed an in vitro mechanical load by stretching the adherent cells. Myocyte stretching increased total cell RNA content and mRNA levels of c-fos. Marked accumulation of c-fos mRNA followed increases in intracellular Na^+ and protein kinase C activation. The accumulation of c-fos mRNA by cardiocyte stretching was suppressed by protein kinase C inhibitors but not by stretch channel blockers. Moreover, myocyte stretching increased inositol phosphate levels, and activation of protein kinase C by phorbol esters stimulated the expression of c-fos. We also examined TGF β expression in the heart. TGF β is known to be stimulated by protein kinase C activation, and the mRNA level of TGF β was increased in in vivo heart by pressure overload. Furthermore, collagen synthesis was stimulated by TGF β in cultured fibroblasts from hearts. These findings suggest that hemodynamic overload may stimulate cardiac hypertrophy and induce cardiac injury (fibrosis) through protein kinase C activation.

SINCE cardiocytes are terminally differentiated, they respond to increased workload by an increase in cell size (hypertrophy), not by an increase in cell number (hyperplasia)! In cardiac hypertrophy induced by hemodynamic overload, the expression of specific genes, as well as an increase in protein synthesis, was induced!⁴ The "immediate early genes", such as protooncogenes and heat shock protein genes, are induced as an early response to pressure overload, and "late responsive genes", such as fetal contractile protein genes and an

atrial natriuretic peptide (ANP) gene, are re-expressed as a later event?²⁻⁴ Recently, norepinephrine has been reported to induce cardiac hypertrophy and specific gene expression in cultured cardiac myocytes via α_1 -adrenergic receptor.^{5,6} Accumulated data shows that an increased workload, either pressure or volume overload, induces cardiac hypertrophy and specific gene expression, but it is not known whether mechanical loading directly regulates gene expression without the participation of humoral or neural factors, or how these mechanical stimuli are converted into intracellular signals of gene regulation.

Since it is now well known that stretching of the ventricular wall during hemodynamic

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Induction of c-fos gene expression by Na^+ influx and C-kinase activation

Fig.1. Induction of c-fos gene expression by Na^+ influx and protein kinase C activation.

Cultured cardiac myocytes were stimulated by the addition of Monensin ($1 \mu\text{M}$), Veratridine ($3 \mu\text{M}$), Ouabain ($2 \mu\text{M}$), TPA (10 ng/ml), and Forskolin ($1 \mu\text{M}$). Cardiac myocytes were stretched by 10% in the Ca^{++} free media ($[\text{Ca}^{++}]_0$ free). After 30 min, RNA was extracted and Northern blot analyses were performed using c-fos DNA as a probe.

overload is the most important mechanical factor for increased protein synthesis,⁷ we cultured neonatal rat cardiocytes in deformable silicone dishes with defined serum-free medium and imposed upon them a mechanical load by stretching adherent cells. Recently, we demonstrated that cardiomyocyte stretching stimulates the expression of c-fos protooncogenes in cultured myocytes.⁸ In the present study, we elucidate the signal transduction pathway of mechanical loading on cardiocytes by using the protooncogene induction by stretching.

It is well known that cardiac hypertrophy is induced as an adaptation to hemodynamic overload, and the heart shows cardiac failure with some injury after long lasting overload. However, the molecular mechanisms of the transition from cardiac hypertrophy to failure remain unknown. We show in this study that some injury (fibrosis) occurs in the hypertrophic stage, and that a peptide growth factor may be involved in this process.

EXPERIMENTAL PROCEDURES

Cell Culture and Cardiocyte Stretching

Primary cultures of cardiac myocytes were prepared from the ventricles of 1 day-old Wistar rats as described previously.⁸ Cells not attached to the pre-plated dishes were plated onto silicone dishes ($2 \times 4 \times 1 \text{ cm}$) at a density of $1 \times 10^5 \text{ cells/cm}^2$ (cardiac myocyte-rich fraction). a non-muscle cell-rich fraction was obtained by pre-plating the cells into silicone dishes for the first hour. The culture medium was changed 24 h after seeding to a serum-free, chemically defined solution consisting of Dulbecco's modified Eagle's medium (DMEM), $1 \mu\text{g/ml}$ of insulin and $5 \mu\text{g/ml}$ of transferrin. Cardiocyte stretching was accomplished as described before,⁸ essentially according to the method of Mann et al.⁹

Measurement of Total RNA

Two days after changing the medium, cardiocytes were stretched by 10% for 24 h. At the termination of the experiment, the cells were rapidly rinsed four times with ice-cold

Inhibition of c-fos gene expression by inhibitors of stretch channel and C-kinase

Fig.2. Inhibition of c-fos gene expression by inhibitors of stretch channel and protein kinase C.

Cardiac myocytes were stretched in the presence of 500 μ M Streptomycin, 100 μ M of Amiloride, 10 μ M H-7 or 10 μ M H-1004. After 30 min, RNA was extracted and Northern blot analyses were performed.

phosphate-buffered saline (PBS, 10 mM sodium phosphate, 0.85% NaCl, PH 7.4). Total cellular RNA was extracted using a lithium urea method¹⁰ and quantitated by spectrophotometry.

Assay for c-fos mRNA

Twenty-four hours after changing the medium, the culture cells which adhered to the silicone dish were stretched for 30 min as described before⁸ or stimulated by the addition of 1 μ M Monensin, 3 μ M Veratridine, 2 μ M Ouabain, 1 μ M Forskolin and 10 ng/ml of 12-0-tetradecanoylphorbol 13-acetate (TPA). Twenty micrograms of total RNA, which was extracted from the cell from four dishes, was fractionated by electrophoresis through 1.2% agarose gels, and Northern blot analysis was performed at least five times as described previously.³ The c-fos DNA was obtained from the Japanese Cancer Research Resources Bank. We used a 0.8 kb AccI fragment of human c-fos as a probe.¹¹ Control hybridizations were carried out using 1.1 kb mouse actin cDNA.¹²

Pharmacological Study of c-fos Induction

In the pharmacological study, cells were stretched in the presence and absence of pro-

tein kinase inhibitors and stretch channel blockers for 30 min. Assay for Inositol Phosphate-Myocytes were incubated for 24 h in DMEM containing myo-[2-³H] inositol (10 μ Ci/ml; New England Nuclear). The dishes were washed several times with 1 ml of assay medium (DMEM containing 25 mM HEPES, 0.25% bovine serum albumin). Myocytes were stretched 10% for 1 or 5 min in the presence of 10 mM LiCl. Control cells were treated in an identical fashion except for stretching. The water-soluble inositol phosphates were separated by column chromatography with Bio Rad AG1-X8 resin as described by Berridge et al.¹³

TGF β Induction by Pressure Overload

Upper abdominal aorta of rats were constricted as described before.¹⁴ RNA was extracted from the heart and Northern blot analyses were performed using murine TGF β oligomer, 5'-GCAGGAGCGCACAATCATGTT-3'.¹⁵

Stimulation of Collagen Synthesis by TGF β

Cells which had adhered to the culture dish after the first hour were used as fibroblast-fich cells. Various concentrations

Stimulation of TGF β Gene Expression by Pressure Overload

Fig.3. Stimulation of TGF β gene expression by pressure overload.

The aortae of 40-day old Wistar rats were constricted and RNA was extracted from the hearts. Northern blot analyses were performed using 5 μ g of polyA RNA and murine TGF β oligomer. mRNA amounts were quantified by densitometry.

of TGF β were added to the dish and [3 H] proline incorporation into collagen was measured as described by Khalil et al.⁶

RESULTS

Passive stretching of cardiocytes for 24 h increased total RNA content by 45% (control, 8.2 ± 0.4 μ g/dish vs stretch, 11.9 ± 0.6 μ g/dish, $n=20$, $p<0.01$). RNA content in the non muscle-rich fraction was not increased by stretching (control, 5.2 ± 0.4 μ g/dish vs stretch, 6.0 ± 0.5 μ g/dish, NS).

We demonstrated previously that cardiomyocyte stretching increased the amount of c-fos mRNA.⁸ To examine the molecular mechanisms by which myocyte stretching stimulates c-fos gene, we added various agents to cultured cardiocytes. Recently, Na⁺ influx has been shown to be induced by cardiac loading and to play an important role in cardiac hypertrophy.¹⁷ Norepinephrine is also reported to induce cardiac hypertrophy and specific gene expression via α_1 -recep-

tors. Thus, we added agents known to increase intracellular Na⁺ or stimulate protein kinase C activity. As shown in Fig. 1, the Na⁺ ionophore, Monensin, and the Na⁺ channel activator, Veratridine, markedly increased c-fos mRNA, and the Na⁺/H⁺ ATPase inhibitor, Ouabain, slightly stimulated c-fos gene expression. The protein kinase C stimulant, TPA, stimulated gene expression, most strongly while the cAMP stimulant, Forskolin, stimulated it very slightly.

As shown in Fig. 2 myocyte stretching stimulated c-fos gene expression. The protein kinase C inhibitor, H-7, strongly inhibited the c-fos mRNA induction caused by stretching, whereas HA-1004 was only weakly inhibitory. H-7 has similar inhibitory effects to H-1004, on cyclic GMP and cyclic AMP-dependent protein kinases but the Ki value for protein kinase C of HA-1004 is higher than that of H-7 (Ki for protein kinase C; H-7, 6.0 μ M vs H-1004, 40 μ M).¹⁸ Staurosporin, a potent and specific protein

Stimulation of Proline Incorporation into Collagen by TGF β in Cardiac Fibroblasts

Fig. 4. Stimulation of proline incorporation into collagen by TGF β in cardiac fibroblasts.

20 μ l of 1-[2,3-³H] proline and various concentrations of TGF β were added and the cells were incubated for 3 h at 37°C. The trichloroacetic acid-precipitated protein was incubated at 37°C for 90 min with or without collagenase III after neutralization. The pellet containing total protein or noncollagen protein was dissolved in formic acid and counted.

kinase C inhibitor, also markedly inhibited c-fos induction at a quite low concentration!¹⁹ After almost complete down-regulation of protein kinase C (5% of control), accumulation of c-fos mRNA by stretching was significantly attenuated!¹⁹ In contrast, the stretch channel inhibitors, Streptomycin and Amiloride, had no significant effects on c-fos mRNA accumulation by stretching. Cardiac stretching also stimulated c-fos gene expression in the absence of extracellular Ca²⁺.

We examined phosphoinositide turnover to ascertain whether myocyte stretching stimulates protein kinase C activity. When cultured myocytes were stretched by 10%, there was a significant increase in the levels of inositol monophosphate (IP₁) and inositol bisphosphate (IP₂). The increases above control values for IP₁ and IP₂ were 55% and 90%, respectively, at 1 min, and 138% and 63%, respectively, at 5 min. Phorbol ester

(TPA) induced c-fos gene expression (Fig. 2), as did 4- β phorbol 12, 13-didecanoate, whereas the 4- α stereoisomer of phorbol 12, 13-didecanoate, which does not stimulate protein kinase C, did not (data not shown).

TGF β was expressed in the normal rat heart and its mRNA levels were increased by aortic banding. The elevation of TGF β mRNA continued to 48h after the imposition of pressure overload (Fig. 3). To examine the effects of TGF β on the heart, we measured amino acid incorporation into collagen using cultured heart-derived fibroblasts. As shown in Fig. 4, TGF β stimulated collagen synthesis in a concentration-dependent manner.

DISCUSSION

Myocyte stretching directly increases the total RNA content of cultured cardiocytes and stimulated protooncogene expression. This "immediate early gene" expression following myocyte loading may be due to protein kinase C activation, but not stretch channel activated Na⁺ influx. Hemodynamic overload induces not only adaptive responses such as α to β transition of myosin heavy chains but also cardiac "injury" such as fibrosis. Pressure overload stimulated TGF β expression in the heart, and TGF β may induce collagen synthesis in the cardiac tissue.

To examine the molecular mechanisms by which mechanical loading affects gene expression, we carried out pharmacological experiments. Pharmacological studies using protein kinase stimulants or inhibitors suggested that protein kinase C activation might be involved in the induction of gene expression by myocyte stretching. Recently, Na⁺ influx through stretch activated channels has been shown to play important roles in cardiac hypertrophy (17); ion fluxes are usually the initial mode of action of many stimuli. In the present study, however, stretch-activated channel blockers had no significant effects on the induction of c-fos stretching, although Na⁺ influx stimulated c-fos expression (Fig. 1, 2). It is well known that the influx of Ca²⁺ through voltage dependent Ca²⁺ channels, leading to formation of the active Ca²⁺-calmodulin complex, is often elevated by extracellular stimuli, and that Ca²⁺ is a major second messenger

Mechanism of Cardiac Hypertrophy and Gene Expression by Hemodynamic Overload

Fig.5. Hemodynamic overload may directly stimulate cardiac hypertrophy and specific gene expression.

Hemodynamic overload (mechanical load) induces myocardial stretch and its stimulus may be converted into phospholipase C activation. The activation of protein kinase C might stimulate protein synthesis and “immediate early” (c-fos and c-myc) and “late response” (actin and myosin) gene expression.

Functions of Protein Kinase C in Cardiocytes

Fig.6. Protein kinase C has various functions in the heart.

The activation of protein kinase C stimulates Na^+/H^+ exchange ANP secretion (27), and Ca^{++} uptake by cardiac sarcoplasmic reticulum through the phosphorylation of phospholamban (25). It might have inhibitory and stimulatory effects on cardiac Ca^{++} channels (23), desensitize adrenergic receptor (24), and also regulate contractile state by causing phosphorylation of troponin subunits (26).

regulating “immediate early gene” expression in excitable cells²⁰ In the stretching of cardiac myocytes, however, Ca^{2+} might not be involved, because c-fos was induced by

stretching even when external Ca^{2+} was completely depleted in the present study (Fig. 2), and the Ca^{2+} response element of the c-fos gene promotor was not sufficient

Mechanisms of Various Events Induced by Hemodynamic Overload

Fig. 7. Various responses to hemodynamic overload in the heart.

Hemodynamic overload may stimulate $TGF\beta$ production and ANP secretion via protein kinase C activation. $TGF\beta$ may induce transition of contractile protein isoforms (29) as an autocrine mechanism and stimulate collagen synthesis through a paracrine mechanism. ANP regulates hemodynamic states by working on vessels and kidneys as a hormone.

for c-fos induction by stretching¹⁹

We examined phosphoinositide turnover to ascertain whether myocyte stretching stimulates protein kinase C activity. Myocardial stretching directly stimulated phosphoinositide turnover in cultured cardiocytes, and activation of protein kinase C by phorbol esters induced c-fos as well as protein synthesis.²¹ Taken together, mechanical stress might directly stimulate protein kinase C activity via phospholipase C activation, and thus, might induce various biochemical events including gene expression (Fig. 5).

Protein kinase C is known to play many important roles in the heart²²⁻²⁷ (Fig. 6). $TGF\beta$ gene has been recently reported to have a phorbol ester-responsive element and to be up-regulated by protein kinase C activation.²⁸ In the present study, $TGF\beta$ mRNA was increased by pressure overload in the heart and $TGF\beta$ stimulated collagen synthesis in heart-derived fibroblasts. $TGF\beta$ was reported to induce isoform transition of contractile protein in the heart.²⁹ $TGF\beta$ protein is known to be expressed in the cardiac myocytes.³⁰ It is tempting to speculate that $TGF\beta$, which is stimulated by hemodynamic overload, may stimulate collagen synthesis

from fibroblasts through a paracrine mechanism and induce isoform switch of contractile proteins in the cardiocytes through an autocrine mechanism. Atrial natriuretic peptide (ANP) is known to play important roles in cardiac function by acting on vessels and kidneys as a hormone. Secretion of ANP is known to be stimulated by protein kinase C activation.²⁷ Therefore, cardiomyocytes might respond to hemodynamic overload in a variety of ways (Fig. 7). Many data have been accumulated suggesting that mechanical loading (hemodynamic overload) in the heart induces expression of both "immediate early genes" such as c-fos and c-my, and "late responsive genes" such as those for β -type myosin heavy chain and skeletal α -actin.²³ The activation of these immediate early genes is likely to play a key role in the transduction of environmental signals into long-lasting changes in cardiac function. Further work is needed, however, to elucidate the molecular mechanisms by which cardiocytes transduce the mechanical stimulus into these biochemical events, and the roles of "immediate early gene" expression in the overloaded heart.

REFERENCES

1. ZAK R: Development and proliferative capacity of cardiac muscle cells. *Circ Res* 1974; **35** (Suppl): 174; **2**: 17
2. IZUMO S, NADAR-GINARD B, MAHDAVI V: Protooncogene induction and reprogramming of cardiac gene expression produced by pressure overload. *Proc Natl Acad Sci USA* 1988; **85**: 399
3. KOMURO I, KURABAYASHI M, TAKAKU F, YAZAKI Y: Expression of cellular oncogenes in the myocardium during the developmental stage and pressure-overloaded hypertrophy of the rat heart. *Circ Res* 1988; **62**: 1075
4. KOMURO I, SHIBAZAKI Y, KURABAYASHI M, TAKAKU F, YAZAKI Y: Molecular cloning of gene sequences from rat heart rapidly responsive to pressure overload. *Circ Res* 1990; **66**: 979
5. STARKSEN NF, SIMPSON PC, BISHOPRIC N, COUGHILIN SR, LEE WMF, ESCOBEDO JA, WILLIAMS LT: Cardiac myocyte hypertrophy is associated with c-myc protooncogene expression. *Proc Natl Acad Sci USA* 1986; **83**: 8348
6. BISHOPRIC NH, SIMPSON PC, ORDAHL CP: Induction of the skeletal α -actin gene in α 1-adrenoreceptor-mediated hypertrophy of rat cardiac myocytes. *J Clin Invest* 1987; **80**: 1194
7. MORGA HE, GORDON EE, KIRA Y, CHUA HL, RUSSO LA, PETERSON CJ, McDERMOTT PJ, WATSON PA: Biochemical mechanisms of cardiac hypertrophy. *Ann Rev Physiol* 1987; **49**: 533
8. KOMURO I, KAIDA T, SHIBAZAKI Y, KURABAYASHI M, KATOH Y, HOH E, TAKAKU F, YAZAKI Y: Stretching cardiac myocytes stimulates protooncogene expression. *J Biol Chem* 1990; **265**: 3595
9. MANN DL, KENT RL, COOPER G: Load regulation of the properties of adult feline cardiocytes: Growth induction by cellular deformation. *Circ Res* 1989; **64**: 1079
10. AUFRAY C, ROUGEON F: Purification of mouse immunoglobulin heavy-chain messenger RNAs from total myeloma tumor RNA. *Eur J Biochem* 1980; **107**: 303
11. VAN STRAATEN F, MULLER R, CURRAN T, BEVEREN CV, VERMA IM: Complete nucleotide sequence of a human c-onc gene: deduced amino acid sequence of the human c-fos protein. *Proc Natl Acad Sci USA* 1983; **80**: 3183
12. MINTY AJ, CARAVATTI M, ROBERT B, COHEN A, DAUBAS P, WEYDERT A, GROS F, BUCKINGHAM ME: Mouse actin messenger RNAs. Construction and characterization of a recombinant plasmid molecule containing a complementary DNA transcript of mouse alpha-actin mRNA. *J Biol Chem* 1981; **256**: 1008
13. BERRIDGE MJ, DAWSON RMC, DOWNES CP, HESLOP RF, IRVINE RF: Changes in the levels of inositol phosphates after agonist-dependent hydrolysis of membrane phosphoinositides. *Biochem J* 1983; **212**: 473
14. KOMURO I, KURABAYASHI M, SHIBAZAKI Y, TAKAKU F, YAZAKI Y: Molecular cloning and characterization of a Ca^{2+} + Mg^{2+} -dependent adenosine triphosphatase from rat cardiac sarcoplasmic reticulum. Regulation of its expression by pressure overload and developmental stage. *J Clin Invest* 1989; **83**: 1102
15. DERYNCK R, JARRET JA, CHEN EY, GOEDDEL DV: The murine transforming growth factor- β precursor. *J Biol Chem* 1986; **261**: 4377
16. KHALIL N, BEREZNAI O, SPORN M, GREENBERG AH: Macrophage production of transforming growth factor β and fibroblast collagen synthesis in chronic pulmonary inflammation. *J Exp Med* 1989; **170**: 727
17. KENT RL, HOOBER JK, COOPER G: Load responsiveness of protein synthesis in adult mammalian myocardium: Role of cardiac deformation linked to sodium influx. *Circ Res* 1989; **64**: 74
18. HIDAKA H, INAGAKI M, KAWAMOTO S, SASAKI Y: Isoquinolinesulfonamides, novel and potent inhibitors of cyclic nucleotide dependent protein kinase and protein kinase C. *Biochemistry* 1984; **23**: 5036
19. KOMURO I, KATOH Y, KAIDA T, SHIBAZAKI Y, KURABAYASHI M, HOH E, TAKAKU F, YAZAKI Y: Mechanical loading stimulates cell hypertrophy and specific gene expression in cultured rat cardiac myocytes: possible role of protein kinase C activation. *J Biol Chem* (in press)
20. SHENG M, MCGADDEN G, GREENBERG ME: Membrane depolarization and calcium induce c-fos transcription via phosphorylation of transcription factor CREB. *Neuron* 1990; **4**: 571
21. FULLER SJ, SUGDEN PH: Protein synthesis in rat cardiac myocytes is stimulated at the level of translation by phorbol esters. *FEBS Lett* 1989; **247**: 209
22. SWANN K, WHITAKER M: Stimulation of the Na/H exchanger of sea urchin eggs by phorbol ester. *Nature* 1985; **314**: 274
23. LACERDA AE, RAMPE D, BROWN AM: Effects of protein kinase C activators on cardiac Ca^{2+} channels. *Nature* 1988; **335**: 249
24. LIMAS CH, LIMAS C: Phorbol ester- and diacylglycerol-mediated desensitization of cardiac β -adrenergic receptors. *Circ Res* 1985; **57**: 443
25. MOVSESIAN MA, NISHIKAWA M, ADELSTEIN RS: Phosphorylation of phospholamban by calcium-activated, phospholipid-dependent protein kinase. Stimulation of cardiac sarcoplasmic reticulum calcium uptake. *J Biol Chem* 1984; **259**: 8029
26. KATOH N, WISE BC, KUO JF: Phosphorylation of cardiac troponin inhibitory subunit (troponin I) and tropomyosin binding subunit (troponin T) by cardiac phospholipid-sensitive Ca^{2+} -dependent protein kinase. *Biochem J* 1983; **209**: 189
27. MATSUBARA H, HIRATA Y, YOSHIMI H, TAKATA S, TAKAGI Y, UMEDA Y, YAMANE Y, INADA M: Role of calcium and protein kinase C in ANP secretion by cultured rat cardiocytes. *Am J Physiol* 1988 **255**: H405
28. KIM SJ, DENHEZ F, KIM KY, HOLT JT, SPORN MB, ROBERTS AB: Activation of the second promoter of the transforming growth factor-beta 1 gene by transforming growth factor-beta 1 and phorbol ester occurs through the same target

- sequences. *J Biol Chem* 1989; **264**: 19373
29. PARKER TG, PACKER SE, SCHNEIDER MD: Peptide growth factors can provoke "fetal" contractile protein gene expression in rat cardiac myocytes. *J Clin Invest* 1990; **85**: 507
30. THOMPSON NL, FLANDERS KC, SMITH JM, ELLINGSWORTH LR, ROBERTS AB, SPORN MB: Expression of transforming growth factor- β 1 in specific cells and tissues of adult and neonatal mice. *J Cell Biol* 1989; **108**: 661