Molecular Mechanism of Cardiac Hypertrophy

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Pressure overload induces cardiac hypertrophy and reexpression of contractile protein isogenes. To ascertain the molecular mechanism of these events, we examined the expression of cellular oncogenes and the early change in the translational activity of specific cardiac mRNA by two-dimensional gel electrophoresis of in vitro translational products. Pressure overload increased the expression levels of c-fos, c-myc, and c-Ha-ras genes. The relative predominance of 8 species out of over 400 translational products was increased by pressure overload while that of 2 translational products was decreased. We cloned four pressure-overload-responsive cDNA clones by differential dot blot hybridization. The expression pattern of each cDNA clone in the pressure-overloaded hearts was similar to that in fetal hearts. To examine whether mechanical stimuli directly induce specific gene expression in the heart, we cultured rat neonatal cardiocytes in elastic silicone dishes and stretched these adherent cells. Myocytes stretching stimulated amino acid uptake and expression of the c-fos gene, which was blocked by protein kinase C inhibitors. These results suggest that there are some early responsive genes in cardiac hypertrophy and that mechanical loading directly stimulates gene expression possibly via protein kinase C activation.

HEMODYNAMIC overload is known to induce cardiac hypertrophy. Since cardiac myocytes lose their ability to replicate soon after birth, cardiac hypertrophy results from enlargement of individual cardiac myocytes.¹ Cardiac hypertrophy is accompanied by increased protein synthesis, total RNA content, and mRNAs for various contractile proteins.²⁻⁴ Many recent studies have demonstrated qualitative changes in the composition of contractile proteins which

accompany quantitative changes in cardiac hypertrophy.⁵⁻⁸ For instance, β type cardiac myosin heavy chain and skeletal α -actin are reexpressed by pressure overload (PO).^{6,7} However, the molecular mechanism by which PO induces cardiac hypertrophy and isoform switching remains unknown.

To extend our knowledge regarding the initial action of PO on hearts, we first examined the existence of cellular oncogenes and the early PO-responsive proteins. Secondly, to examine whether mechanical stimuli are directly coupled to specific gene expression, we cultured neonatal rat cardiocytes in elastic silicone dishes with defined, serum-free medium, imposed mechanical

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stimuli in vitro by stretching adherent cardiocytes and examined c-fos gene expression.

METHODS

Animal Preparation

To produce PO, male Wistar rats, weighing 180–200g, were anesthetized with diethylether and the upper part of the abdominal aorta was constricted with a hemoclip as described previously.⁹ To investigate the developmental changes, hearts of 12-, 15-, and 18-day-old embryos, 5-day-old neonates, and 40- and 200-day-old adults were examined.

RNA Preparation and Northern Blot Analysis

Total cellular RNA was extracted from the left ventricles and Northern blot hybridization was carried out as described previously.⁹ Eight oncogene probes, human c-myc, exon III, EcoRI/Clal¹⁰; v-fos, BglII/PvuII¹¹; v-Ha-ras, BalI/SalI¹²; v-erbA, PstI/PstI¹³; v-erbB, BamHI/EcoRI¹³; v-sis, PstI/PstI¹⁴; v-myb, SalI/SalI¹⁵; v-src, PvuII/PvuII¹⁶ were used as probes. The human c-myc probe was a generous gift of Dr. Sekiya. Other viral oncogenes were obtained from the Japanese Cancer Research Resources Bank.

Cell-free Translation and Analysis of In Vitro Translation Products

RNA-dependent cell-free translation was performed using rabbit reticulocyte lysate (Amersham) and [³⁵S]methionine essentially according to Dillmann et al.¹⁷ The reactions were started by the addition of 1 µg of poly(A⁺) RNA and incubated at 30°C for 60 min. Samples containing equal amounts of radioactivity were subjected to two-dimensional gel electrophoresis as described previously.¹⁸ After electrophoresis, the gels were prepared for fluorography, dried and exposed to Kodak XAR-5 film at –70°C.

Screening of the cDNA Library

A total of 1500 single plaques of rat heart cDNA library (purchased from Clontech Laboratory Inc.) were isolated with sterile toothpicks, grown individually in microtiter wells, and replicated onto nylon membranes. Five µg of poly(A⁺) RNA isolated from sham-operated and 8h-PO hearts was labeled using reverse transcriptase. For differential plaque hybridization, one set of filters was hybridized with ³²P-labeled probe complementary to mRNA which was extracted from sham-operated rat hearts, and

the other set of filters was hybridized with an equivalent probe derived from 8h-PO rat hearts. Hybridization and autoradiography were performed as described previously.⁹ Phage DNA that gave a different signal between duplicate membranes was purified according to Maniatis et al.¹⁹

Cell Culture

Primary cultures of cardiac myocytes were prepared from the ventricles of day-old Wistar rats essentially according to the method of Simpson et al.²⁰ To reduce the number of contaminating non-muscle cells, dissociated cells were pre-plated into 100-mm culture dishes in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal calf serum for 1 h. We made elastic culture dishes (2 × 4 × 1 cm) by vulcanizing liquid silicone rubber consisting of methyl vinyl polysiloxane and dimethyl hydrogen silicone resin using platinum as a catalyst. The bottom of the dish is 1 mm thick and it is highly transparent. Cells not attached to the pre-plated dishes were plated into laminin-coated (20 µg/ml) silicone dishes at a field density of 1 × 10⁵ cells/cm² (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 DMEM, 1 µg/ml of insulin and 5 µg/ml of transferrin. At this point, more than 90% and 20% of the cells were beating in the cardiac myocyte-rich fraction and the non-muscle cell-rich fraction, respectively.

Amino Acid Incorporation into Protein

After 2 days in the serum-free medium, the culture dishes were stretched by 10% and 1 µCi/ml [¹⁴C] phenylalanine was added for 30 min prior to 2 and 8 h after stretch. Stretching cardiocytes was accomplished in the following manner. To stretch the deformable dishes, we made stretch frames (3 × 5 × 1.5 cm) of fluorocarbon plastic. The silicone dish was fixed to the sides of the stretch frame. The stretch frame was designed to be mechanically expanded by turning a horizontally mounted thumb screw. A 10% change in the length of the dish resulted in about a 10% change in the length of the cell along a single axis. Total trichloroacetic acid insoluble radioactivity was determined by liquid scintillation counting as described previously.²¹

Fig.1. Functions of protooncogenes (A) and expression of their transcripts in the rat heart after aortic constriction (B).

A. Functions of several protooncogenes are shown.

B. The aorta of male Wistar rats was constricted with a hemoclip. The rats were killed at the indicated times after the operation and RNA was extracted from the heart. 3 μ g of poly (A⁺) RNA was separated on 1.2% agarose gel, blotted onto a nylon membrane and hybridized to three oncogene probes. The autoradiograms were exposed for 5 days with intensifying screen at -70°C .

Assay for c-fos mRNA

Twenty four hours after changing the medium, the culture cells which were adherent to the silicone dish were stretched as described above for various periods of time. At the termination of stimulation, the cells were lysed in situ, and total cellular RNA was extracted.

Construction of Chimeric Plasmid and Transfection Experiments

A 1.4-kb NaeI fragment containing upstream

sequences and the promotor of the fos gene was isolated by agarose gel electrophoresis and sub-cloned into the HindIII site at the 5' end of the chloramphenicol acetyltransferase (CAT) coding sequence in the plasmid pSV0CAT by means of HindIII linkers. Myocytes were transfected with 1 μ g of DNA per dish using the CaPO₄ precipitation method. The medium was changed after 24h and cardiocytes were stimulated by stretching or 10% serum, 48h after transfection. After 2h, cell extracts were prepared and assayed for

Fig.2. Two-dimensional gel electrophoresis of in vitro translational products. Translational products of control rats (0°), rats with imposition of pressure overload for 8h (8°) were electrophoresed. 1, 3, 5–10, increased predominance in pressure-overloaded samples; 2, 4, decreased predominance in pressure-overloaded samples; 0, pressure overload-insensitive products used as an internal control; LC1, myosin light chain 1; LC2, myosin light chain 2; TM, tropomyosin; A, actin. Horizontal axis, isoelectric focusing from pH 3.5 (right) to pH 7.5 (left); vertical axis, sodium dodecylsulfate polyacrylamide gel electrophoresis.

CAT activity as described by Gorman et al.²²

RESULTS

RNA Blot Hybridization Analysis of c-oncogenes in Pressure-overloaded Cardiac Hypertrophy.

Cellular oncogenes have been presumed to play a role in growth control (Fig. 1A). Northern Blot analysis showed that 3 oncogenes, c-fos, c-myc and c-Ha-ras, were expressed at appreciable

levels in PO rat hearts (Fig. 1B). The increase of the expression of c-fos and c-myc related sequences were recognized from as early as 30 min and 2h, respectively, after the operation, peaked at 8h and gradually decreased thereafter to the baseline at 48h. Some c-Ha-ras related sequences were expressed in sham operated hearts, and increased gradually by pressure overload.

Fig.3. Northern blot analysis of pressure-overload-responsive clones during acute pressure overload and developmental stage.

Aortas of male Wistar rats were constricted with a hemoclip and rats were killed at indicated times after operation. RNA was extracted from pressure-overloaded left ventricles (A) or hearts of 12-, 15-, and 18-day-old embryos (Fetus 12d, 15d, and 18d), 5-day-old neonates (Neo.), and 40- and 200-day-old adults (Adult 40d, and 200d) (B). Poly1(A⁺) RNA (3 μ g) was separated on 1.2% agarose gel, blotted onto a nylon membrane, and hybridized to pressure-overload-responsive cDNA clones. Autoradiograms were exposed for 2 to 5 days with intensifying screens at -70°C .

Analysis of In Vitro Translation Products

Figure 2 shows representative two-dimensional patterns of translational products encoded by cardiac RNA isolated from 0 and 8 h after PO. The predominance of 8 translational products^{1,3,5-10} was significantly increased while that of 2 products^{2,4} was decreased by acute PO. Although there was a great difference in the translational products between adult and fetal

hearts, 6 out of 8 translational products, of which the expression levels were rapidly increased by PO, were also recognized in fetal hearts (data not shown).

Molecular Cloning of Pressure-overload-responsive mRNAs

Northern blot analysis was performed to discard false positive. A total of 4 PO-responsive

Fig.4. Northern blot analysis of c-fos mRNA by stretching cultured cardiocytes.

A. Neonatal rat cardiocytes cultured in a deformable dish were stretched by 10% for various periods of time. B. Cells were stretched in the presence or absence of the indicated inhibitors (each 10 μ g/ml) for 30 min. RNA was extracted, run on a gel and transferred to the filters. The filters were hybridized with the c-fos probe.

clones designated pPO-1, 4, 5, and 6, survived, and the size of each cloned cDNA was 1.5, 2.2, 2.0, and 2.5 kb, respectively. The size of their corresponding mRNAs ranged from about 1.5 kb to 7.6 kb by Northern blot analysis. The expression level of pPO-1 was increased at 4h after PO, peaked at 8h, and decreased to the basal level at 24h. In pPO-4, not only the expression level was increased but also another molecular weight-different mRNA appeared from 8h after aortic constriction. The expression level of pPO-5 was decreased gradually and that of pPO-6 was transiently decreased between 8 and 12h operation.

Amino Acid Incorporation Into Protein

Passive stretching of cardiocytes stimulated amino acid incorporation into proteins. The amino acid incorporation was significantly in-

creased 2h after stretching (stretch, $5.2 \pm 0.2 \times 10^3$ cpm/dish vs control, $4.1 \pm 0.2 \times 10^3$ cpm/dish, $p < 0.05$), and the stimulation was maintained for 8h (stretch, $7.4 \pm 0.4 \times 10^3$ cpm/dish vs control, $4.5 \pm 0.3 \times 10^3$ cpm/dish, $p < 0.01$).

c-fos Induction by Myocyte Stretching

Northern blot analysis using a c-fos-specific DNA revealed that myocyte stretching induced the accumulation of c-fos mRNA, although there were no signals at the anticipated 2.2-kilobase transcripts in the control. Accumulation of c-fos mRNA was detected as early as 15 min after the passive stretching of cardiocytes and reached a maximal level at 30 min, followed by a decline to undetectable levels (Fig. 4A). The c-fos induction by stretching was inhibited by H-7, while H-1004 was only weakly inhibitory. Staurosporin, a potent protein kinase C inhibitor, also inhibited c-fos induction (Fig. 4B).

Transfection Experiments

To examine whether the increased mRNA levels of c-fos gene by stretching were regulated at the transcriptional level or post-transcriptional level, we transfected plasmids containing chimeric c-fos gene into primary cultures of neonatal cardiac myocytes and measured CAT activity. As shown in Fig. 5, when pSV0fosCAT, which contained the 5' c-fos sequence, was introduced into the system, myocardial stretching caused more than a 7-fold increase in CAT activity, but there was little activity without stretching.

DISCUSSION

We demonstrated the existence of genes, including some protooncogenes, of which the expression levels were rapidly changed by acute pressure overload in the rat heart and isolated 4 PO-responsive cDNA clones. Mechanical stimuli directly induced specific gene expression as well as protein synthesis without the participation of humoral factors. This protooncogene stimulation was blocked by protein kinase C inhibitors. Increased CAT activity of transfected c-fos fusion gene indicated that the specific c-fos gene expression by stretching was regulated at least partially at the transcriptional level.

Many data have been accumulated confirming that PO induces not only quantitative changes but also qualitative changes in the heart.⁵⁻⁸ Fetal isoforms of the contractile proteins were reexpressed by PO. For example, α to β myosin

Fig.5. Cardiac-stretch responsiveness of CAT activity in transfected cells.

A. Construction of recombinant plasmid, pSV0fosCAT, containing the c-fos upstream and promoter region linked to the CAT coding sequence. A 1.4-kb NaeI fragment was cloned into the unique HindIII site in the pSV0CAT vector.

B. Cultured cardiocytes were stimulated by stretching or 10% serum, 48h after transfection with the plasmids, pSV0fosCAT.

heavy chain transition in rat hearts is induced by PO.^{5,6} The question remains as to how PO induces the cardiac hypertrophy and isoform switching. In this acute PO model, the increment of heart weight was detected from 2 or 3 days after aortic constriction and the mRNA of β myosin heavy chain increased from 24h (data not shown). Thus, we examined more rapidly responsive proteins, since the possibility might exist that these proteins play an important role in the subsequent events.^{23,24} We isolated 4 PO-responsive clones. Although their expression patterns in PO hearts differed from each other, they were similar to those in fetal hearts. Cardiocytes lose their ability to replicate after birth; however, cell hypertrophy of adult myocytes and cell division of fetal myocytes may share a common mechanistic pathway.

When the heart was exposed to hemodynamic overload, specific genes, such as protooncogenes and hsp70 gene, were induced and fetal type contractile proteins were reexpressed.^{7,8,25} Recently, many reports have demonstrated that

humoral factors, such as thyroid hormone and norepinephrine, induce gene expression in cultured cardiocytes.²⁶⁻²⁸ But because of difficulties in imposing work-overload on cardiocytes in vitro, it is not known whether mechanical stimuli directly cause these biochemical responses. In the present study, we imposed mechanical stimuli by stretching rat neonatal cardiocytes cultured in elastic dishes under defined, serum-free medium and demonstrated that mechanical loading in cardiocytes induced specific gene expression as well as cell hypertrophy.

We demonstrated that the expression of c-fos gene was stimulated by aortic stenosis in the heart in vivo, and by myocyte stretching in vitro. These results suggest that hemodynamic overload itself is one of the main factors to stimulate the expression of c-fos gene in the heart, in vivo. The protein which it encodes, Fos, has been localized to the nucleus and is believed to play an important role in regulating the subsequent expression of other genes.²⁹ Quite recently, Fos was elucidated to bind to the TPA-responsive

elements of some genes and to activate their gene transcription, in cooperation with the transcriptional factor AP-1.^{30,31} The c-fos gene expression by stretching was blocked by protein kinase C inhibitors and Bishopric et al suggested that the activation of protein kinase C may stimulate early developmental gene reexpression in cardiocyte hypertrophy.²⁷ These results and observations lead us to speculate that some early responsive gene products like Fos may stimulate other subsequent gene expressions in the heart under conditions of hemodynamic overload. Further work is needed to elucidate the roles of these early PO-responsive genes, including protooncogenes, in cardiac hypertrophy.

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