Communication

Stretching Cardiac Myocytes Stimulates Protooncogene Expression*

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Recently cellular protooncogenes have been found to
be induced as an early response to pressure overload in
cardiac hypertrophy. To examine whether mecha-
nical stimuli directly induce specific gene expression in
the heart, we cultured rat neonatal cardiocytes in elas-
tic silicone dishes and stretched these adherent cells.
Myocyte stretching stimulated expression of the pro-
tooneogene, c-fos, in a stretch length-dependent man-
er, followed by an increase in amino acid incor:
poration into proteins. c-fos mRNA levels were enhanced
within 15 min by cardiocyte stretching, peaked at 30
min, and declined to undetectable levels by 240 min.
In the presence of cycloheximide, a greater increase in
c-fos mRNA was seen by stretching. The transfected
chloramphenicol acetyltransferase gene linked to up-
stream sequences of the fos gene including its promoter
was also activated by stretching cardiac myocytes.
These results suggest that mechanical loading directly
regulates gene transcription without the participation of
humoral factors in cardiocytes.

Since cardiac myocytes cannot divide later in life, they
respond to the demand for increased mass by an increase in
cell size (hypertrophy), not by an increase in cell number
(proliferation) (1). In cardiac hypertrophy, the expression of
specific genes, such as protooncogenes and fetal-type genes of
contractile proteins, was induced as well as an increase in
protein synthesis (2-4). Many factors, including hemody-
namic overload and humoral factors, have been reported to
induce cardiac hypertrophy (5, 6). Recently, norepinephrine
has been elucidated to induce cardiac hypertrophy (5, 6). Recently, norepinephrine
was also activated by stretching cardiac myocytes.

EXPERIMENTAL PROCEDURES

Cell Culture—Primary cultures of cardiac myocytes were prepared
from the ventricles of 1-day-old Wistar rats essentially according to the
method of Simpson et al. (9). Trypsinization was performed with
0.05% tryspin-EDTA at 37 °C for 10 min. Cells from the first
were discarded, and the sequence was repeated until all tissue
was dissociated (about five times). Cardiocytes were maintained at
37 °C in humidified air with 5% CO2 in order to achieve a final
medium pH of 7.35 - 7.40. To reduce the number of contaminating
nonmuscle cells, dissociated cells were preplated in 100-mm culture
dishes in Dulbecco's modified Eagle's medium with 10% fetal calf
serum for 1 h. We purchased liquid silicone rubber from Shin-Etsu
Chemical Co., Ltd., Tokyo, Japan and made elastic culture dishes (2
× 4 × 1 cm) by vulcanizing liquid silicone rubber consisting of
methylvinyl 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 because of no inorganic filler in either compo-
nent. Cells not attached to the preplated dishes were plated into
aminin-coated (20 µg/ml) silicone dishes at a cell density of
1 × 10^6 cells/cm² (cardiac myocyte-rich fraction). A nonmuscle cell-rich
fraction was obtained by preplating 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 mod-
ified Eagle's medium, 1 µg/ml insulin, and 5 µg/ml transferrin. At
this point, more than 90% and less than 20% of the cells were beating
in the cardiac myocyte-rich fraction and the nonmuscle cell-rich
fraction, respectively. We removed hearts from 30 to 50 neonatal rats
at one time and dissociated and plated cells into 30-50 dishes. Stretch
and control experiments were carried out simultaneously with the
same pool of cells in each experiment.

Amino Acid Incorporation into Protein—After 2 days in the serum-
free medium, the culture dishes were stretched by 10%, and 1 µCi/ml
[^3]C]Ileucylalanine was added for 30 min prior to 2 and 8 h after
stretch. Stretching cardiocytes was accomplished in the following
manner essentially according to the method of Mann et al. (10). For
the stretch of deformable dishes, we made stretch frames (3 × 5 ×
1.5 cm) of fluorocarbon plastic. The stretch frames were removed
from the incubator and placed on a 37 °C warming plate in a sterile
laminar flow hood. Then, the silicone dish was fixed to the sides of
the stretch frame. The stretch frame was designed so that it could be
mechanically expanded by turning the horizontally mounted thumb
screw; turning the screw caused the frame to expand, thereby unifi-
cally increasing the length of the attached dish. It was then returned
to the 37 °C incubator. Stretch frames were kept in 150-µm poly sty-
rene Petri dishes while maintained in the incubator. This entire
procedure was completed within 30 s, during which time the pH and
temperature of the medium did not change appreciably. Since the
bottom of the silicone dish was transparent, we could measure the
degree of stretch of each cell. Although most neonatal rat cardiocytes
were irregularly shaped, not rod-shaped, the resting length of the cells
was increased parallel to the axis of deformation by the same percent
length as the dish reported before (10). Thus, a 10% change in the
length of the dish resulted in a 9.3 ± 0.9% (n = 100) change in the
length of the cell along a single axis. Control cells were treated
simultaneously in an identical fashion except for stretching so that
dynamic overload directly induces specific gene expression
without humoral factors remains unknown.

We have reported that the expression of protooncogenes such as c-myc and c-fos genes was markedly stimulated in rat
hearts by aortic stenosis (4). But, since the previous study
was performed on hearts in vivo, we could not rule out the
participation of humoral factors. In the present study, 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 stimuli in vitro by stretching adherent
cardiocytes, and examined protooncogene expression.

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The manipulation did not make a difference in the other conditions such as the temperature, CO₂ content, or pH of the medium between control and stretched cells. At the termination of the experiment, the cells were rapidly rinsed four times with ice-cold phosphate-buffered saline (10 mM sodium phosphate, 0.85% NaCl, pH 7.4) and incubated for 20 min on ice with 1 ml of 5% trichloroacetic acid. Total trichloroacetic acid-insoluble radioactivity of each dish was determined by liquid scintillation counting. We repeated the experiment three times in duplication.

**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. The northern blot techniques were performed as described previously (4). The blots were quantitated by scanning densitometry. 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 (12). Control hybridizations were formed as described previously (4). The blots were quantitated by liquid scintillation counting. We repeated the experiment three times.

**RESULTS**

As shown in Fig. 1A, passive stretching of cardiocytes stimulated amino acid incorporation into proteins. The amino acid incorporation was significantly increased 2 h after stretching, and the stimulation was maintained for 8 h. Northern blot analysis using a c-fos-specific DNA revealed that myocyte stretching reproducibly induced the accumulation of c-fos mRNA, although there was no signal at the anticipated 2.2-kilobase transcripts in the control (Fig. 1B). 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. 1A). Stretch length dependence in the induction of c-fos mRNA was exhibited. Stimulation of c-fos gene expression was recognized by 5% stretching, and maximal stimulation was obtained by 20% stretching (relative mRNA levels: 5% stretching, 15 ± 5%; 10% stretching, 89 ± 9%; 20% stretching, 100%; mean ± S.E., n = 5) (Fig. 2B).

Treatment with the protein synthesis inhibitor, cycloheximide, alone produced a rapid increase in c-fos mRNA. The combined treatment with myocyte stretching and cycloheximide resulted in greater levels of c-fos mRNA expression than treatments with either stimulant alone (Fig. 3A). This protooncogene was abundantly expressed in cardiac myocyte-rich fraction (myocytes) rather than in non-muscle cell-rich fraction (non-myocytes) (Fig. 3B, relative mRNA levels: myocytes, 100% versus non-myocytes, 26 ± 6%, n = 6), suggesting that the stimulation of c-fos gene expression by stretching occurred in cardiac myocytes.

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. 4, the pSV0CAT plasmid showed very little CAT activity, but there was little activity without stretching (relative acetylated chloramphenicol levels: stretch, 100% versus control, 12 ± 3%, n = 7). When the pSV2CAT vector, which...
cultured cardiocytes were treated with 20 pg/ml cycloheximide for 30 min or stretched by 10% for 30 min with or without 20 pg/ml cycloheximide. B, the cardiac myocyte-rich fraction (myocyte) and non-muscle cell-rich fraction (non-myocytes) were stretched by 10% for 30 min. Northern blot analysis was performed as described under “Experimental Procedures.” The autoradiogram of the same filter with the plasmids pSVOCAT (a, b), pSVOfosCAT (c, d), and pSV2CAT (e, f) into primary 1-day-old rat heart myocytes. Autoradiograms of thin layer chromatograms using the assay mixture after hybridization with c-fos probe (A), and parallel samples of stained 18 S ribosomal RNA (B) are shown below the autoradiograms to demonstrate the integrity of RNA samples. Cyclo, cycloheximide-treated cells; C, control cells; S, stretched cells.

FIG. 3. Northern blot analysis of c-fos mRNA by stretching cultured cells in the absence or presence of cycloheximide. A, cultured cardiocytes were treated with 20 pg/ml cycloheximide for 30 min or stretched by 10% for 30 min with or without 20 pg/ml cycloheximide. B, the cardiac myocyte-rich fraction (myocyte) and non-muscle cell-rich fraction (non-myocytes) were stretched by 10% for 30 min. Northern blot analysis was performed as described under “Experimental Procedures.” The autoradiogram of the same filter hybridized with c-actin probe (A), and parallel samples of stained 18 S ribosomal RNA (B) are shown below the autoradiograms to demonstrate the integrity of RNA samples. Cyclo, cycloheximide-treated cells; C, control cells; S, stretched cells.

FIG. 4. Cardiocyte 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 stretched by 10% 48 h after transfection with the plasmids pSV0CAT (a, b), pSV0fosCAT (c, d), and pSV2CAT (e, f) into primary 1-day-old rat heart myocytes. Autoradiograms of thin layer chromatograms using the assay mixture after 1 h of incubation are demonstrated. a, c, e, control cells; b, d, f, stretched cells.

activity of the transfected c-fos fusion gene indicated that the specific c-fos gene expression by stretching was regulated at least partially at the transcriptional level and that the stretch-response element was located in the 5'-flanking DNA of the c-fos gene.

When the heart was exposed to hemodynamic overload, specific genes such as protooncogenes and hsp70 gene were induced and fetal type genes of contractile proteins were re-expressed (3, 4, 16). Recently, many reports have demonstrated that humoral factors such as thyroid hormone and norepinephrine induce gene expression in cultured cardiocytes (2, 8, 17). But because of difficulties in imposing work overload on cardiocytes in vitro, it is unknown 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 have reported that the expression of the c-fos gene was stimulated by aortic stenosis in in vivo heart studies (4). In the present study, the expression of c-fos protooncogene was markedly induced by mechanical loading without the participation of humoral factors. These results suggest that hemodynamic overload itself is one of the main factors to stimulate the expression of the 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 (18). Quite recently, Fos was elucidated to bind to the 12-O-tetradecanoyl-phorbol-13-acetate-responsive elements of some genes and to activate their gene transcription in cooperation with the transcriptional factor AP-1 (19, 20). Cardiocytes respond to hemodynamic overload by reinduction of fetal type contractile proteins as well as cell hypertrophy. Bishopric et al. (8) suggested that the activation of protein kinase C may stimulate early developmental gene re-expression in cardiac hypertrophy. 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 physiological roles of protooncogenes in cardiac hypertrophy.

REFERENCES
1. Zak, R. (1974) Circ. Res. 35, Suppl. 2, 17-26
2. Starkensen, N. F., Simpson, P. C., Bishopric, N., Coughlin, S. R., Lee, W. M., Escobedo, J. A., and Williams, L. T. (1986) Proc. Natl. Acad. Sci. U. S. A. 83, 3348-3350
3. Isumo, S., Nadal-Ginard, B., and Mahdavi, V. (1988) Proc. Natl. Acad. Sci. U. S. A. 85, 339-343
4. Komuro, I., Kurabayashi, M., Takaku, F., and Yasaki, Y. (1988) Circ. Res. 62, 1075-1079
5. Cooper, G., Kent, R. L., Uboh, C. E., Thompson, E. W., and Marino, T. A. (1985) J. Clin. Invest. 75, 1403-1414
6. Simpson, P., McGrath, A., and Savion, S. (1982) Circ. Res. 51, 781-801
7. Simpson, P., Bishopric, N., Coughlin, S., Karszner, L., Ordahl, C., Starkensen, N., Tsao, T., White, N., and Williams, L. T. (1986) J. Mol. Cell. Cardiol. 18, Suppl. 5, 45-88
8. Bishopric, N. H., Simpson, P. C., and Ordahl, C. P. (1987) J. Clin. Invest. 80, 1194-1199
9. Simpson, P., and Savion, S. (1982) Circ. Res. 50, 101-116
10. Mann, D. L., Kent, R. L., and Cooper, G. (1989) Circ. Res. 64, 1079-1090
11. Auffray, C., and Rougeon, F. (1980) Eur. J. Biochem. 107, 303-314
12. Fyffe, D. F., Muller, R., Curran, T., Beveren, C. V., and Verma, I. M. (1983) Proc. Natl. Acad. Sci. U. S. A. 80, 3183-3187
13. Minty, A. J., Caravatti, M., Robert, B., Cohen, A., Daubas, P.,
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Weydert, A., Gros, F., and Buckingham, M. E. (1981) J. Biol. Chem. 256, 1008–1014
14. Gorman, C. M., Moffat, L. F., and Howard, B. H. (1982) Mol. Cell. Biol. 2, 1044–1051
15. Izumo, S., Lompre, A.-M., Matsuoka, R., Koren, G., Schwartz, K., Nadal-Ginard, B., and Mahdavi, V. (1987) J. Clin. Invest. 79, 971–978
16. Schwartz, K., de la Bastie, D., Bouveret, P., Oliveiro, P., Alonso, S., and Buckingham, M. (1986) Circ. Res. 59, 551–555
17. Gustafson, T. A., Markham, B. E., Bahl, J. J., and Morkin, E. (1987) Proc. Natl. Acad. Sci. U.S.A. 84, 3122–3126
18. Marx, J. L. (1987) Science 237, 854–856
19. Chiu, R., Boyle, W. J., Meek, J., Smeal, T., Hunter, T., and Karin, M. (1988) Cell 54, 553–560
20. Sassone-Corsi, P., Lamph, W. W., Kamps, M., and Verma, I. M. (1988) Cell 54, 553–560


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