Induction of c-fos mRNA Expression by Pure Pressure Overload in Cultured Cardiac Myocytes

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SUMMARY

Mechanical stress by pressure overload due to hypertension or valvular heart disease such as aortic valve stenosis induces cardiac hypertrophy. It has been well established that the mechanical stretch of cardiac myocytes in vitro induces hypertrophic responses such as the expression of immediate early response genes including c-fos. However, it remains uncertain whether the mechanical forces due to pure atmospheric pressure can induce similar responses in cardiac myocytes. We thus cultured rat neonatal cardiac myocytes in an atmospheric pressure chamber apparatus and determined the effects of pure pressure stress on c-fos gene expression. Pressures greater than 80 mmHg enhanced c-fos mRNA after 30 minutes. These results suggest that pure atmospheric pressure overload can also induce early hypertrophic responses in cardiac myocytes. (Int Heart J 2007; 48: 359-367)

Key words: Experimental technique, Hypertrophy, Immediate early gene, Molecular experiment, Pressure stress

CARDIAC hypertrophy is an independent risk factor for cardiovascular events and death.1) Hemodynamic overload caused by hypertension or valvular heart disease such as aortic valve stenosis produces cardiac hypertrophy. The mechanical stretch of cultured cardiac myocytes has been shown to stimulate hypertrophic responses including the expression of immediate early genes and fetal contractile protein genes as well as protein synthesis.2-4) This model of stretching cells in vitro has been described as mechanical stress-induced cardiac hypertrophy.5,6) However, it is possible that the stretch of myocytes cultured on deformable silicone dishes differs from the stress due to pressure overload. Mechanical single stretch produces static, but not dynamic, forces on cardiac myocytes. In contrast, hemodynamic forces include not only static stretching but also dynamic pressure stress. Our previous studies have demonstrated that pure pressure overload could

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induce DNA synthesis and mitogen-activated protein kinase (MAPK) family activation in cultured human aortic smooth muscle cells.\(^7\)

In the present study, we examined whether mechanical overload under pure atmospheric pressure could induce the expression of c-fos mRNA, which is one of the hallmarks of hypertrophic molecular alterations seen in cardiac myocytes. We used a pressure-loading apparatus that was capable of directly applying various levels of pure pressure up to 160 mmHg on cultured cells in order to determine the effects of pure pressure stress without inducing morphological changes.\(^8\)

**METHODS**

**Cardiac myocyte isolation and culture:** Primary cultures of cardiac myocytes were prepared from 1-day-old Wistar rats according to the methods of Perez-Terzic, et al, with some modifications.\(^9\) In brief, excised hearts were separated into ventricular and atrial tissues, and the ventricles were cut into 4 equal parts with scissors in \(1 \times\) Ads buffer (in mmol/L: NaCl 116; HEPES 20; NaH\(_2\)PO\(_4\) 10.8; glucose 5.6; KCl 5.4; MgSO\(_4\) 0.8, pH 7.40) prior to enzymatic digestion. The ventricular tissue was subjected to serial enzymatic digestion (5 digestions of 20 minutes each) with collagenase (65 units/mL, Worthington Biochemical Corporation)-pancreatin (2.5 mg/mL, Sigma) solution, using a stirring water bath (Wheaton) at 41°C. The supernatant was centrifuged (6 minutes, 700 rpm) and the pellet with myocytes was suspended in fetal bovine serum (Gibco) and incubated (37°C, 5% CO\(_2\)). After collecting the cells by centrifugation (6 minutes, 700 rpm), they were resuspended in \(1 \times\) Ads buffer (0.15 mL/heart) and subjected to discontinuous 2-layer Percoll (Sigma) gradient centrifugation (30 minutes, 3000 rpm) in order to segregate the myocytes from nonmyocytes. Cardiac myocytes that were present in the first layer from the bottom were collected, suspended in \(1 \times\) Ads buffer, and centrifuged twice (10 minutes, 700 rpm). Pellets were suspended in plating medium, 68% DMEM (Sigma); 17% medium 199 (Sigma); 10% horse serum (Gibco); 5% fetal bovine serum (Gibco); 2mM L-glutamine (Sigma); 100 units/mL penicillin/streptomycin (Gibco); and 0.2% insulin-transferrin-selenium media supplement (Gibco). Cells were plated onto 35-mm collagen type-1 coated dishes (Iwaki, Japan) in vitro and incubated in the serum-containing medium (37°C, 5% CO\(_2\)).\(^{10}\) After 24 hours, the media was replaced by serum-free media (DMEM and Media 199, 4:1), supplemented with 2mM L-glutamine and 100 units/mL penicillin/streptomycin for 48 hours. All procedures in this study were performed in accordance with the Institutional Animal Care and Use Committee of the Hokkaido University Graduate School of Medicine.
Pure pressure-loading apparatus: Pure atmospheric pressure stress was applied to the myocytes using the pure pressure-loading apparatus we described previously, with some modifications (Figure 1). The chamber allows air or nitrogen gas to be pumped in to raise the internal pressure (maximum at 160 mmHg), and it can be sealed tightly by placing several clamps at the bottom edge. Internal pressure levels were monitored with an aneroid barometer during the experiments. The compression chamber was established in the incubator and kept at 37°C, and a digital thermometer was mounted in the incubator to monitor the exact internal temperature. In the following series of experiments, the chamber was kept at 37°C, and the partial pressures of pO₂ and pCO₂ were theoretically

![Figure 1](image-url)
preserved as constants according to the Boyle-Gay-Lussac law. The pH in the culture medium was kept at a constant level (7.41 ± 0.02) during the experiments. It was not possible to monitor the actual morphological changes of pressurized cells in our system set-up. However, our preliminary light microscopic investigations confirmed no apparent changes in cell size or morphology during and after pressurization.\(^7\)

After 48 hours of serum starvation, the cardiac myocytes were placed in the pure pressure-loading apparatus in the medium containing 10 mM HEPES, and exposed to various levels of atmospheric pressure (0 mmHg, 80 through 160 mmHg) at 37°C.

**Semiquantitative RT-PCR analysis of c-fos gene:** Total RNA was isolated from cardiac myocytes using TRIzol Reagent (Gibco) according to the manufacturer’s instructions. After ethanol precipitation, RNA samples were resolved in 20 µL of diethylpyrocarbonate water. Concentration was photometrically quantified at 260 nm. The ratio of optical density at 260 and 280 nm was over 1.6 in all cases. One µg of total RNA from each sample was reverse-transcribed according to a method described previously.\(^11\) Semiquantitative RT-PCR analysis of c-fos gene was performed using the aforementioned diluted cDNA and Advantage 2 PCR kit (Clontech). The primers (synthesized by Hokkaido System Science Inc., Sapporo, Japan) were originally designed. PCR was carried out as follows: 5 cycles of 94°C for 30 seconds, 57°C for 30 seconds and 72°C for 40 seconds, then 28 cycles of 94°C for 30 seconds, 62°C for 30 seconds and 72°C for 40 seconds, plus 1 cycle of 72°C for 5 minutes. PCR of GAPDH was performed according to methods described previously.\(^12,13\) PCR products were subjected to electrophoresis on 1.0% agarose gels. Ethidium bromide-stained bands were viewed and photographed under UV light. Densitometric analysis was performed using Scion image β4.02 win software (http://www.scioncorp.com). Within a given experiment, the densitometric values were normalized using GAPDH concurrently run within the same gel.

**Statistical analysis:** Data are expressed as the mean ± SEM obtained from at least 3 independent experiments. Differences were evaluated for significance using the 2-tailed Student’s \(t\) test. \(P\) values < 0.05 were considered to be statistically significant.

**RESULTS**

After 48 hours of serum starvation, dishes on which isolated cardiomyocytes were plated were placed in the pressure chamber apparatus. Nitrogen gas was pumped into the chamber in order to increase atmospheric pressure within the chamber, and cultured cardiomyocytes were compressed under various pres-
sures between 80 and 160 mmHg (in 20 mmHg increments) for various periods of time. As shown in Figure 2, atmospheric compression of the cardiac myocytes stimulated the expression of c-fos gene, which is one of the immediate early response genes. Semiquantitative RT-PCR analysis revealed that accumulation of c-fos gene was detected after 60 minutes and reached maximal levels after 90 minutes. The cardiac myocytes were then compressed for 60 minutes, under various pressures, in order to determine the threshold of pure atmospheric pressure in acceleration of c-fos gene production. It was detected at the compression of 80 mmHg, and remained increased up to 160 mmHg (Figure 3).

Figure 2. Time course of c-fos gene expression in cardiac myocytes by pure pressure overload. Representative semiquantitative RT-PCR analysis of c-fos gene expression (A) and its summary data (B). Cardiac myocytes were compressed by hydrostatic pressure of 160 mmHg. c-fos gene was detected at 30 minutes and reached maximal levels at 60 minutes. Values are the mean ± SEM. * P < 0.05 for difference from the 0 value.
The results of the present study demonstrate that pure pressure overload to cultured cardiac myocytes using an atmospheric pressure chamber apparatus could directly induce the molecular hypertrophic response of c-fos gene expression. This finding is consistent with a previous study that reported increased aortic pressure could increase protein synthesis in the Langendorff heart.\(^{14}\)

Cardiac hypertrophy can be induced in response to various stresses such as pressure and/or volume overload\(^{15-18}\) as well as neurohumoral factors including basic fibroblast growth factor, insulin-like growth factors, endothelin, and angiotensin II \textit{in vitro}.\(^{19-24}\) These studies have demonstrated that the mechanical stretch of cardiac myocytes can directly and independently induce hypertrophic

\textbf{Figure 3.} c-fos gene expression in cardiac myocytes under various pure pressure overloads. Representative semiquantitative RT-PCR analysis of c-fos gene expression (A) and its summary data (B). Cardiomyocytes were compressed for 60 minutes at various hydrostatic pressures. Values are the mean ± SEM. * \(P < 0.05\) for difference from the 0 value.
responses, including specific gene expression.\textsuperscript{3,4,25}

The passive stretch of cardiac myocytes cultured on silicone membranes has been demonstrated to induce c-fos mRNA expression and protein synthesis.\textsuperscript{5} However, this longitudinal passive stretch differs from the mechanical forces which are imposed on cardiac myocytes \textit{in vivo}. Cardiac myocytes are not only stretched passively but are also exposed to pressure stress during the contraction. This pressure overload is more important as a stimulus to induce cardiac hypertrophy \textit{in vivo}. Therefore, it must be crucial to determine whether pure pressure overload to cardiac myocytes can induce the hypertrophic responses. In fact, the contraction, but not the stretch, of the cells has been shown to activate Ras and inactivate Rap1 in mouse fibroblastic L-929 cells and human embryonic kidney-derived 293 cells.\textsuperscript{26} Moreover, previous studies have demonstrated that pure pressure overload could promote DNA synthesis in cultured smooth muscle cells.\textsuperscript{7,27}

We have demonstrated in the present study using continuously applied compressive pressure overloading to compress cardiac myocytes\textsuperscript{28} that pure pressure stress could also induce the early response gene expression. Using an original pressure-loading apparatus, we were able to determine the effects of mechanical stress on cardiac myocytes due to various pressure levels from 0 to 160 mmHg. This experimental system allowed us to investigate the cellular responses to pure pressure overload under conditions without neurohumoral effects and cellular deformation.\textsuperscript{8} As shown in Figure 3, c-fos mRNA accumulation seems to decrease under pressures exceeding 160 mmHg, although the difference between its expression induced by pressures of 140 mmHg and 160 mmHg was not significant in our experiments. It is possible that dynamic pressure stress induces only acute induction of c-fos gene and that passive stretch chronically induces hypertrophic responses by cardiomyocytes.

In summary, the present study has demonstrated that pure pressure stress could stimulate intracellular biochemical signals similar to those induced by passive stretch. Therefore, cardiac myocytes have mechanosensing mechanisms, including mechanosensors and/or intracellular signaling pathways, activated by pure atmospheric pressure.

\textbf{REFERENCES}