The Studies of Cell Damaging and Cell Growth Factors which Induce Cardiomyopathy

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We demonstrated that phosphatidylinositide-specific phospholipase C (PLC) activity was greater in cardiomyopathic hamster hearts (BIO 14.6 and BIO 53.58) than in hamster controls (F1b). Inositol trisphosphate (IP_3) production was markedly greater in both of the cardiomyopathic hamsters, BIO 14.6 and BIO 53.58. We have also determined the sarcoplasmic reticulum (SR) function of heart. Calcium uptake into SR markedly increased in BIO 14.6. On the other hand, it significantly decreased in BIO 53.58 compared with F1b. It is well known that IP_3 stimulates calcium release from SR. In BIO 14.6, calcium release from SR stimulated by IP_3 increased, but its effect decreased in BIO 53.58 compared with F1b. These results suggest that PI response may produce high intracellular calcium levels in both BIO 14.6 and BIO 53.58 myocytes. In addition, in the BIO 53.58 hamster the sarcoplasmic reticulum deteriorate in function. It was concluded from these results that a prolonged high intracellular calcium level may lead to the death of BIO 53.58 myocytes.

The expression of angiotensinogen mRNA was observed in the hamster heart. There was no differences in its expression level between F1b, BIO 14.6 and BIO 53.58. There was no effect of ages on its expression in these hamster hearts. We have also determined the distribution of angiotensinogen in these hamsters. At 4 weeks of age, the immunohistochemical study revealed that angiotensinogen was widely distributed in subendocardium in these hamsters. There was no difference in its distribution between F1b, BIO 14.6 and BIO 53.58. But at 20 weeks old of age its immunoreactivity decreased in BIO 53.58. There was no effect of age on its reactivity in F1b and BIO 14.6.

We have detected angiotensinogen in heart, but its role is still not clear. A further study should be done to clarify its role in cardiac hypertrophy and cell damage.

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SYRIAN cardiomyopathic hamsters (BIO 14.6 and BIO 53.58) display hereditary abnormalities of the cardiac and skeletal muscles which are inherited as an autosomal recessive trait! BIO 14.6 cardiac involve-

ment results in initial myocardial hypertrophy that is followed by cardiac dilatation and death from congestive heart failure? It is thought the cardiomyopathic hamster provides a useful model of human cardiac diseases such as hypertrophic cardiomyopathy^{3,4}

Many hormones and neurotransmitters stimulate the breakdown of plasma membrane inositol phospholipids by the media-

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Fig.1. Heart weight/body weight (mg/g) of cardiomyopathic (hatched bar, upper pane; BIO 53.58, lower panel; BIO 14.6) and age-matched F1b (open bar) hamsters. * $p < 0.05$, ** $p < 0.001$ compared with F1b.

tion of specific receptors^{5,6-8} This so-called phosphatidylinositol (PI) turnover pathway generates two second messengers, inositol-(1, 4, 5)-trisphosphate (IP_3) and sn-1, 2-diacylglycerol (DAG)^{9,10} DAG stimulates membrane-bound phospholipid-dependent, Ca^{2+} -dependent protein kinase C¹¹ while IP_3 releases Ca^{2+} from stores in the sarcoplasmic reticulum^{12,13} The physiological significance of this PI-turnover pathway is not still clear in any mammalian cell system. It is controversial whether IP_3 stimulates Ca^{2+} release from SR in cardiac myocytes.

The renin-angiotensin (RA) system plays a role in the regulation of blood pressure and of fluid and electrolyte homeostasis. Renin cleaves angiotensinogen (ang-n) to produce angiotensin I, which is then converted by angiotensin-converting enzyme (ACE) to angiotensin II, the most active pressor substance known. They form the major circulating RA system¹⁴ Recently, molecular cloning and sequence analysis of complementary DNAs (cDNAs) coding for rat ang-n¹⁵ and human ang-n^{16,17} have provided an opportunity to study ang-n at the messenger RNA (mRNA) level. In the heart, angiotensin II appears to play an important role in cardiac hypertrophy¹⁸ A clinical study reported that ACE inhibitor (ACEI),

Fig.2. Phospholipid hydrolysis stimulated by NE. Phospholipid hydrolysis was determined as described in Materials and Methods. BIO 14.6 (A), BIO 53.58 (B). △; phosphatidylcholine, ○; phosphatidylinositol, □; phosphatidylethanolamine, ▲; lysophosphatidylcholine, ●; lysophosphatidylinositol, ■; lysophosphatidylethanolamine

which inhibits the conversion of angiotensin I to angiotensin II, caused regression of cardiac hypertrophy¹⁹ Thus, the tissue RA system may be implicated in cardiac hypertrophy and other cardiac disorders in man. However, little interest has been focused on the possibility of local synthesis of ang-n in human organs, especially in the heart.

In this report, we determined the role of PI-response and cardiac RA system in inducing myocardial cell damage and cardiac myocyte hypertrophy in dilated hamster hearts and hypertrophic cardiomyopathic hamsters.

MATERIALS AND METHODS

Experimental Protocol

Experiments were carried out using male hypertrophic cardiomyopathic hamsters (BIO 14.6) aged 5, 10, 20 and 30 weeks, and age-matched male dilated cardiomyopathic hamsters (BIO 53.58) (Bio Breeders, Fitchburg, Massachusetts)⁴ F1b hamsters were used as control and there were 10 animals in each age group.

Cell preparation

Cardiac myocytes from BIO 14.6, BIO 53.58 and F1b hamsters were prepared in phosphate buffer (PB) according to a previously reported method²⁰ and then cultured

Fig.3. Effects of norepinephrine stimulation on IP₃ release (A) BIO 14.68 (hatched bar), (B) BIO 53.58 aged 5–30 weeks (hatched bar) and F1b (open bar). *p<0.05 compared with age-matched F1b, **p<0.001 compared with age-matched F1b.

in Ham's F-10 medium with 10% fetal calf serum (FCS) until use. Freshly prepared cells were maintained at 37°C in a humidified 5% CO₂/95% air atmosphere. Cells were then subcultured for assay in 35-mm dishes at 3 × 10⁵/dish in 1 ml of PB containing 1 mM CaCl₂ and used within 2 h.

Phospholipase C activity

For the determination of the cellular phospholipase C activity, cells (1 × 10⁵) were first labeled with 5 μCi of [³H]myoinositol, [³H]choline chloride, or [³H]ethanolamine. Labeling was performed for 24 h in PB with 0.3% FCS, after which cells were washed three times with PB. Cells were then incubated with the indicated concentrations of NE, 5 mM 2, 3-diphosphoglyceric acid (2, 3-DPG; this concentration inhibited the dephosphorylation of IP₃ and IP₄ by 98%), and 10 mM LiCl for the indicated periods in the presence of 1 μM metoprolol, and then

Fig.4. Ca²⁺-uptake into Sarcoplasmic reticulum. Ca²⁺ uptake was determined as described in Materials and methods.

terminated with chloroform/methanol (2: 1, v/v). Phospholipids were fractionated by TLC using a chloroform/methanol/acetic acid/water solvent system (50: 30: 8: 4, v/v). For the separation of polyinositol-phosphatides, the aqueous phase was applied to an AG1 × 8 column in format form (100–200 mesh; Bio-Rad), and inositol phosphates were separated by an ammonium gradient system (0.2–1.2 M) plus 0.1 M formic acid²¹. For a more detailed analysis, including the separation of inositol phosphate isomers, samples were filtered and separated by high-performance liquid chromatography (Whatman Partisil 10 SAX anion-exchange column with a guard column) using a gradient of ammonium formate and phosphate²². The release of IP₃ was also determined by using an IP₃-binding protein system (myoinositol-(1, 4, 5)-trisphosphate assay system, Amersham, UK).

Calcium release from sarcoplasmic reticulum

Left ventricles were minced and homogenized for 60 seconds by a polytron homogenizer (PT-19, Kinematica, Switzerland).

Fig.5. Ca²⁺-release from Sarcoplasmic reticulum stimulated by IP₃. Ca²⁺-release was determined as described in Materials and methods.

land) in 5 volumes (vol/wt) of 0.1 M Tris-HCl (pH 7.4).

The homogenate was centrifuged at 1,000 g for 10 min, and the resultant supernatant was centrifuged at 14,000 g for 20 min to remove mitochondria. The supernatant was then centrifuged at 45,000 g for 30 min according to previously published methods²³. The pellet was washed twice with the same buffer and suspended in 0.6 M KCl and 10 mM tris (hydroxymethyl) methyl-2-aminomethane-sulfonic acid (TES) pH 7.4. The suspension was centrifuged at 45,000 g for 30 min. The resultant pellet was suspended in 150 mM KCl and 1 mM TES pH 7.1 and used for each assay as sarcoplasmic reticulum. The yield of sarcoplasmic reticulum protein in BIO hearts was similar to F1b hearts (0.82 ± 0.1 and 0.84 ± 0.2 mg/g, respectively). Na⁺-K⁺-ATPase activity and cytochrome oxidase activity in sarcoplasmic reticulum fraction were <1% of the total activities and were similar in two groups.

⁴⁵Ca²⁺ (4 μ Ci) was loaded into freshly prepared sarcoplasmic reticulum by incubat-

ing 400 μ g of sarcoplasmic reticulum in a final reaction mixture of 400 μ l in cytosolic buffer (KCl 120 mM, NaCl 10 mM, KH₂PO₄ 1 mM, NaHCO₃ 5 mM, HEPES 10 mM, pH 7.1), MgCl₂ 5 mM, and ATP 5 μ M for 10 min. The final free Ca²⁺ concentration was calculated according to the method of Fabiato and Fabiato²⁴ and adjusted to 1 μ M. In Ca²⁺ release experiments, IP₃ was added to the 100 μ l-aliquot of ⁴⁵Ca²⁺-loaded sarcoplasmic reticulum in the presence of 1 mM EGTA, 10 mM LiCl and 5 mM 2, 3-diphosphoglycerate (2, 3-DPG; this concentration of 2, 3-DPG inhibited the dephosphorylation of IP₃), and immediately mixed with the reaction medium. Ca²⁺ release started from this time. The experiments were carried out at 37°C. After the indicated incubation periods, the incubation mixture was filtered through a 0.45 μ Millipore filter, washed three times with 3 ml of cytosolic buffer, and counted in 5 ml of Ready Gel. The zero-time Ca²⁺ content of the sarcoplasmic reticulum was obtained by diluting the original incubate and immediately filtering it through a Millipore filter. The total sarcoplasmic reticulum calcium content was 388 ± 22 nmol/mg protein. There was no differences between the two groups for the calcium content of sarcoplasmic reticulum.

Northern blott hybridization

RNA was denatured with formaldehyde at 65°C for 15 min and was subjected to 1.0% agarose gel electrophoresis (Seakem GTG agarose, FMC Bioproducts). RNA was transferred to a nylon membrane (Hybond-N⁺, Amersham International, UK) by capillary action with 10×SSC over night and was fixed with 0.05 M NaOH, then washed with 5×SSPE. The membranes used for transfer were prehybridized at 42°C for 1 h, in a solution consisting of 50% formamide, 5×SSPE, 5×Denhardt's solution, 0.1% SDS, 200 μ g/ml denatured salmon sperm DNA, and 5% dextran sulfate; then they were hybridized over night at 42°C in the same buffer, to which radio-labeled ang-n cDNA (generous gift from A. Fukamizu, University of Tsukuba) had been added. After hybridization, the membranes were exposed to X-ray film (Kodak X-OMAT, Eastman Kodak Company, Rochester, NY) for 5 days. And the same membranes were hybri-

Fig.6. Expression of angiotensinogen mRNA.

dized α -actin cDNA after rehybridization. The angiotensinogen mRNA expression was also determined by the ribonuclease protection assay (RPA) method.

Immunohistochemical techniques

The ABC method was applied to the paraffin-embedded tissues after fixation with 10% formalin and 4% paraformaldehyde/0.1 M phosphate buffer. Briefly, after deparaffinization with xylene and hydration with ethanol, the sections were treated with goat serum and 1% H_2O_2 methanol for quenching of endogenous peroxidase activity. Then the sections were incubated with the primary antibody of ang-n (generous gift from A. Fukamizu, University of Tsukuba) at dilutions of 1: 800 at 4°C over night. After washing with PBS, they were incubated with a second antibody (biotinylated goat anti-mouse immunoglobulins; Biogenex, San Ramon, CA, USA). Then the sections were reacted with the ABC (Elite ABC kit, Vector Laboratories Inc., Vectastain) and visualized with 3-3'-diaminobenzidine tetrahydrochloride.

Statistical Analysis

Six experiments in triplicate were analyzed in all the studies, and results were expressed as the mean $\pm$ SEM. Statistical significance was determined using the previously de-

scribed method (ANOVA) and taking $p < 0.05$ as the limit of significance.

RESULTS

The heart weight/body weight was higher at 5–20 weeks of ages in BIO 14.6 hamsters than in F1b hamsters. There was no difference at 30 weeks of age between both hamsters. On the other hand, it was lower in all age groups of BIO 53.58 hamsters than in F1b hamsters (Fig. 1).

Polyphosphoinositide-specific phospholipase C activity was studied. Myocardial cells isolated from 10-week-old BIO 14.6, BIO 53.58, and F1b hamsters were incubated with the indicated concentrations of NE and 1 μ M metoprolol in the presence of 10 mM LiCl and 5 mM 2, 3-DPG for 90 sec. NE induced the hydrolysis of PI, but lysoPI accumulation was not observed (Fig. 2). This indicates that phospholipase has substrate specificity towards PI, and suggests that phospholipase C was activated but not phospholipase A_2 . There was no significant difference between the BIO 14.6 and BIO 53.58 hamsters. As shown in Fig. 3, the PIP_2 -PLC activity of isolated cells was markedly enhanced by NE stimulation in both types of cardiomyopathic hamster. IP_3 release was enhanced by NE from the age of 5 weeks to 30 weeks. At this stage, the release of IP_3 in

Fig. 7. Immunoreactivity of angiotensinogen.

response to NE was significantly activated in both BIO 14.6 and BIO 53.58 hamsters. This acceleration of PI-turnover may affect Ca^{2+} flux into cells. As previously reported, the basal intracellular calcium concentration without NE stimulation was higher in BIO 53.58 hamsters than in BIO 14.6 and F1b hamsters²⁵. Intracellular calcium levels in BIO 53.58 hamsters aged 5–20 weeks were significantly higher than those of age-matched BIO 14.6 hamsters, but there were no significant differences between two types of cardiomyopathic hamsters at 30 weeks of

age. The sarcoplasmic reticulum (SR) function of cardiomyopathic hamster hearts were investigated. As shown in Fig. 4, calcium uptake into SR increased in BIO 14.6, but decreased in BIO 53.58. The addition of IP_3 to $^{45}\text{Ca}^{2+}$ -preloaded sarcoplasmic reticulum from F1b, BIO 14.6 and BIO 53.58 in a medium containing $1\ \mu\text{M}$ free Ca^{2+} induced a transient release of Ca^{2+} and achieved a maximum effect at $10\ \mu\text{M}$ IP_3 for 1 min-incubation at 37°C . The Ca^{2+} release from sarcoplasmic reticulum in BIO 14.6 was significantly higher than in F1b. On the other hand, calcium release from SR decreased in BIO 53.58 (Fig. 5).

The expression of cardiac angiotensinogen mRNA was determined by northern blot analysis and RPA. Cardiac angiotensinogen mRNA was expressed in these animals. There was no difference in its expression between these hamsters (Fig. 6).

By immunohistochemical study of angiotensinogen, in the heart, intense immunoreactivity was found in the myocardial cells of the atrium. The myocardial cells in the atrium that were adjacent to the endocardium exhibited a stronger reaction than did those near the epicardium. Immunoreactive cells were present in a mottled pattern. In the ventricle, an immunopositive reaction was recognized in the subendocardial layer, but the intensity was weaker than that in the atrium. At 20 weeks of age, the immunoreactivity of angiotensinogen decreased in BIO 53.58. There were no differences with age between F1b and BIO 14.6 (Fig. 7).

DISCUSSION

In our experiment, the accumulations of polyphosphoinositides after stimulation with NE were significantly enhanced in cardiac myocytes of cardiomyopathic hamster. These results suggest the possibility that the enhanced phosphatidylinositol-(4, 5)-bisphosphate- IP_3 - Ca^{2+} pathways and the DAG-PKC pathway may increase protein synthesis in the BIO 14.6 heart. However, this hypothesis does not explain how myocardial cell damage occurs in BIO 53.58 hamsters. In both animals, the phosphatidylinositol metabolism and the polyphosphoinositide metabolism are enhanced, but

one develops cardiac hypertrophy and the other develops cardiac dilatation. This difference may be partly explained by the cytosolic Ca^{2+} concentration as reported elsewhere.²⁵ The cytosolic Ca^{2+} concentration was quite high in BIO 53.58 hamsters aged 4–30 weeks, but in BIO 14.6 hamsters, it increased at 30 weeks of age. As mentioned above, an increase in the PI-response may raise the cytosolic Ca^{2+} level, but in BIO 14.6 hamsters aged 4–20 weeks it was still lower than in BIO 53.58 hamsters. These results suggest that the intracellular calcium handling is seriously disturbed from a very young age in BIO 53.58 hamsters. On the other hand, it appears to be well-preserved in the early stages of hypertrophic cardiomyopathy in BIO 14.6 hamsters. Our results supported this hypothesis. Calcium uptake into sarcoplasmic reticulum decreased in BIO 53.58 hamsters compared to BIO 14.6 and F1b hamsters.²⁶ These results show that α_1 -adrenergic receptor stimulation may cause intracellular calcium overload because of deterioration of sarcoplasmic reticulum function in BIO 53.58 hamsters.

In the cardiac renin-angiotensinogen system, angiotensin II stimulates PI response in myocytes. In these cardiomyopathic hamsters, angiotensinogen is present in the heart. The immunoreactivity of angiotensinogen in BIO 53.58 decreased, but its role is not still clear. A further study should be done to clarify its role on cardiac hypertrophy and cell damage.

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