A Mechanism of Catecholamine Tolerance in Congestive Heart Failure

— Alterations in the hormone sensitive adenylyl cyclase system of the heart —

KAZUSHI URASAWA, M.D., KATSUHIKO SATO, M.D., YASUMI IGARASHI, M.D.
HIDEAKI KAWAGUCHI, M.D., AND HISAKAZU YASUDA, M.D.

It is well known that failing hearts show diminished responsiveness (desensitization) to catecholamines. In this study, 2 different animal models were used to investigate the alterations in the hormone sensitive cardiac adenylyl cyclase system in a congestive heart failure. In the first model, cardiomyopathic Syrian hamsters (BIO53.58), we found reduced activity of the catalytic protein of adenylyl cyclase; this reduction was more prominent in an older animals (28-week-old vs 16-week-old). At both ages, the amount of inhibitory GTP-binding protein (G_i) was markedly increased in BIO53.58 compared to control healthy hamsters. Moreover the increased G_i was shown to be fully functional in the inhibitory pathway of the adenylyl cyclase system. In the second model, chronically norepinephrine-infused rats, we found a decrease in β -adrenergic receptor density at an early stage of injection (3 days), while the activity of catalytic protein decreased beyond 14 days of injection, and the amount of G_i increased after 7 days of injection. These results suggest that increased plasma catecholamine concentrations in the setting of congestive heart failure might be a major trigger for qualitative and quantitative alterations observed in various components of cardiac adenylyl cyclase system, and that GTP-binding proteins and catalytic protein of adenylyl cyclase are involved in the mechanism of desensitization especially after chronic *in vivo* stimulation of adrenergic receptors.

(*Jpn Circ J* 1992; **56**: 456–461)

VARIOUS diseases and clinical settings can induce congestive heart failure (CHF). As such, it is unlikely that a single mechanism can explain the cardiac dysfunction observed in CHF. However, CHF patients (or animals) share several similar features: reduced cardiac contractility, increased sympathetic nerve activity, and activation of several hormonal systems (eg, renin-angiotensin system, vasopressin, and atrial natriuretic factor) to compensate

for the impaired cardiac function. In this setting, cardiac receptors, GTP-binding proteins, and their effector molecules (adenylyl cyclase, phospholipase C, several ion channels) are inevitably exposed to chronically elevated neurohormonal stimulation. A widely recognized concept is that a chronic increase in such stimulation can desensitize target cell receptors and post-receptor signal transducing pathways! It is therefore likely that in CHF one or more types of desensitization contributes to a reduction in signal transducing efficiency in order to maintain intracellular homeostasis. To date, several laboratories have reported a decrease in β -

Key words:

Heart failure
Adenylyl cyclase system
GTP-binding protein
Desensitization

Department of Cardiovascular Medicine, Hokkaido University School of Medicine Sapporo 060, Japan
Mailing address: Kazushi Urasawa, M.D. Department of Pharmacology, M-0636 University of California San Diego La Jolla, CA92093 USA

Fig.1. Alteration in inhibitory GTP-binding protein (G_i) of BIO53.58 and F1B.

A: Quantitative measurement of G_i . Cholate extracted membrane fractions were incubated with 32 P-NAD and preactivated pertussis toxin. Radiolabeled products were separated by rapid filtration through nitrocellulose membranes, and quantitated by liquid scintillation counter.

B: Functional assay of G_i . Crude membrane fractions were incubated with $10 \mu\text{M}$ forskolin in the presence of various concentration of GTP γ S. Results were normalized to $10 \mu\text{M}$ forskolin-stimulated cyclase activity (without GTP γ S).

adrenergic receptor (βAR) density in CHF patients and model animals^{2-5,6}. Recently, reports of several studies have indicated that the inhibitory GTP-binding protein (G_i) can be increased in CHF patients^{7,8} and animal models⁹. Although direct evidence for a change in the catalytic protein of adenylyl cyclase has not been available, limited information has suggested a reduced catalytic activity in terminally failing hearts¹⁰. In this study, we have assessed changes of βAR , GTP-binding protein and catalytic protein using 2 different animal models with an emphasis on defining the kinetics for the desensitization process of adenylyl cyclase system.

Fig.2. Forskolin-stimulated adenylyl cyclase activity of BIO53.58 and F1B.

Crude membrane fractions were incubated with $100 \mu\text{M}$ forskolin at 30°C for 15 min. The radioactive product, ^{32}P -cAMP, was separated from the substrate α - ^{32}P -ATP using ion-exchange column (Dowex AG50W, Bio Rad, USA), and alumina column, then counted in liquid scintillation counter.

MATERIALS AND METHODS

Materials: 1) Cardiomyopathic Syrian hamsters, BIO53.58, were obtained from BIO Research Laboratory (USA). In this study, animals in 2 different ages (16- and 28-week-old) were used to examine the relation between severity of CHF and changes in various parameters. Hamsters in F1B were used as healthy controls (4 animals for each group). 2) A nonhypertensive dose of norepinephrine (NE), $0.25 \mu\text{g/kg/min}$, was injected into Wistar Kyoto rats subcutaneously using implanted osmotic pumps (model 2002, Alzet, USA). Prior to this study, we conducted experiments to determine an optimal NE dose. We found that a higher dose of NE (such as $1 \mu\text{g/kg/min}$) could increase blood pressure, promote cardiac hypertrophy, and reduce body weight, thus indicating potent cardiovascular and metabolic effects of NE. In order to focus on more localized effects of NE on the cardiac adenylyl cyclase system, we used a lower (non-hypertensive) dose ($0.25 \mu\text{g/kg/min}$). In separate experiments, we measured plasma NE concentrations after 7 days of injection; control group ($n=6$) $144.2 \pm 28.5 \text{ pg/ml}$, NE group ($n=6$) $1646.7 \pm 252.6 \text{ pg/ml}$. The NE injection was

Fig.3. Density of β -adrenergic receptor of Norepinephrine infused rats. The density (Bmax) of β -adrenergic receptor was obtained by Scatchard analysis of ^{125}I -cyanopindolol binding experiments.

maintained for 3 different durations, 3, 7 and 14 days (6 animals in each group). Physiological saline was injected into 6 Wistar Kyoto (WKY) rats for 14 days using the same technique, and this group was used as control.

Membrane preparation: A crude membrane fraction was prepared as described previously¹¹. Briefly, hearts were minced in ice-cold potassium phosphate buffer (pH 7.5), homogenized by Polytron, and centrifuged at 1,250 g for 30 min. The resultant supernatant fractions were centrifuged again at 100,000 g for 1h, and the pellet fractions were suspended in the potassium-phosphate buffer and stored at -80°C until use. Protein concentration of the crude membrane fraction ranged from 1 to 2 mg/ml.

β AR binding experiments: ^{125}I -cyanopindolol (ICYP, Amersham, USA) was used for the β AR binding experiments as described previously¹¹. The results were analyzed by Scatchard plot, and receptor density (Bmax) and dissociation constant for ICYP (Kd) were obtained.

ADP-ribosylation of GTP-binding proteins: Cholate extracted membrane fractions were incubated with ^{32}P -NAD (Dupont NEN, USA) and preactivated bacterial toxin (cholera toxin for stimulatory GTP-binding protein, G_s , or pertussis toxin G_i) as described previously¹². Radiolabeled products were filtered through nitrocellulose membranes (A025A25, Advantec, Japan), and counted in scintillation counter.

Fig.4. Alteration in GTP-binding proteins of Norepinephrine infused rats.

A: Quantitative measurement of stimulatory GTP-binding protein (G_s) and inhibitory GTP-binding protein (G_i). Cholate extracted membrane fractions were incubated with ^{32}P -NAD and preactivated cholera toxin or pertussis toxin. Radiolabeled products were separated by rapid filtration through nitrocellulose membranes, and quantitated by liquid scintillation counter.

B: Results were normalized by control values of each GTP-binding proteins.

Assay of adenylyl cyclase activity: Forskolin ($100\mu\text{M}$)-stimulated cyclase activity was measured by conventional column methods using ^{32}P -ATP (Dupont NEN, USA) as substrate and ^3H -cAMP (Dupont NEN, USA) as calibration standard¹³.

Protein assay: Protein concentration was determined by Amido-black stain method¹⁴. Bovine serum albumin was used as the protein standard.

Statistical analysis: All experiments were performed in duplicate, and multiple observations were reported as the mean $\pm$ SD. Results were analyzed by *t*-test with $p<0.05$ considered to be statistically significant.

Fig. 5. Forskolin-stimulated adenylyl cyclase activity of Norepinephrine infused rats. Crude membrane fractions were stimulated by 100 μ M forskolin, and adenylyl cyclase activity was determined by a standard column chromatographic method (see MATERIALS AND METHODS).

RESULTS

GTP-binding proteins of BIO53.58: The amount of G_i , measured by pertussis toxin-catalyzed ADP-ribosylation, was significantly increased in BIO53.58 compared to FIB at both ages (16-week-old; FIB 0.51 ± 0.05 , BIO 0.95 ± 0.25 , 28-week-old; FIB 0.57 ± 0.13 , BIO 1.37 ± 0.33 pmol/mg protein) (Fig. 1a). Forskolin-stimulated cyclase activity was inhibited by the addition of GTP γ S to a greater extent in 28-week-old BIO53.58 than in age-matched FIB (Fig. 1b). The amount of G_s , measured by cholera toxin-catalyzed ADP-ribosylation was similar between BIO53.58 and FIB at both ages (data not shown).

Forskolin-stimulated cyclase activity of BIO53.58: The activity of catalytic protein, assessed by forskolin-stimulated cyclase activity, was markedly decreased in 16-week-old BIO53.58 (FIB 240.0 ± 31.2 , BIO 145.7 ± 13.7 pmol cAMP/mg min). This decrease was more prominent in the 28-week-old group (FIB 269.9 ± 6.5 , BIO 91.1 ± 18.2 pmol cAMP/mg/min) (Fig. 2).

β AR of NE-infused rats: The density of β AR was significantly reduced in all 3 treated groups (70.7 ± 6.2 , 51.4 ± 5.8 , 41.5 ± 7.2 and 49.5 ± 8.2 fmol/mg for control, 3-, 7- and 14-day group, respectively) (Fig.

3). The dissociation constant of β AR for 125 I-cyanopindolol was essentially the same in all four groups (100.1 ± 38.8 , 84.7 ± 12.6 , 91.9 ± 9.3 and 91.3 ± 18.8 pM for control, 3-, 7- and 14-day group).

GTP-binding proteins of NE-infused rats: The amounts of G_i were 6.02 ± 0.84 , 6.17 ± 0.74 , 8.08 ± 1.25 and 7.37 ± 1.18 (pmol/mg) for control, 3-, 7- and 14-day group, respectively, and significantly increased after 7-day treatment. There was no significant difference in the amount of G_s among the 4 groups (Fig. 4).

Forskolin-stimulated cyclase activity in NE-infused rats: The catalytic activity of NE-infused rats was significantly decreased at 3 days of treatment, and maintained at a reduced level until 14 days of treatment (235.1 ± 47.7 , 174.1 ± 24.7 , 167.4 ± 31.2 and 128.1 ± 35.7 pmol cAMP/mg/min for control, 3-, 7- and 14-day group) (Fig. 5).

DISCUSSION

Cardiomyopathic Syrian hamsters, BIO53.58, are characterized by progressive cardiac dilatation and early death with CHF within one year¹⁵. The most prominent difference from strain BIO14.6, which has been widely used as an animal model for cardiomyopathy, is that BIO53.58 does not develop cardiac hypertrophy before CHF stage. Because of this characteristic feature, BIO53.58 may be a more suitable model than BIO14.6 to study dilated cardiomyopathy and CHF of humans.

In this study, BIO53.58 had an increased amount of G_i and a reduced catalytic activity. Several laboratories have reported an increase of G_i in human CHF^{7,8}. Böhm et al reported that the increase of G_i in dilated cardiomyopathic hearts was associated with reduced basal and GTP analogue-stimulated cyclase activity, and reduced responsiveness to isoprenaline¹⁶. We also found that the increased G_i in BIO53.58 was functional and produced a greater activity in inhibiting adenylyl cyclase (Fig. 1b).

There are several reports which indicate normal forskolin-stimulated cyclase activity in failing hearts^{8,17}. However, Harding et al recently reported that atrial myocytes isolated from NYHA class IV patients show reduced amplitude in maximal contractile

response to forskolin!¹⁰ This would suggest that the catalytic protein is also impaired in terminally failing hearts and such impairment could contribute to post-receptor dysfunction. Additionally, CHF induced by rapid ventricular pacing in pigs is also associated with a decrease of β AR density and reduction of catalytic activity (H. Kirk Hammond, personal communication). In our study, the catalytic activity of BIO53.58 was decreased even at the early stage of CHF (16-week-old), and was impaired to a greater extent in the older age group (28-week-old). In conjunction with the fact that increase of G_i in 28-week-old BIO53.58 was more prominent than in 16-week-old BIO53.58 (Fig. 2), these alterations observed in post receptor components might be related to the severity of CHF.

In the second model that we studied, a low dose ($0.25 \mu\text{g/kg/min}$) of NE was chronically injected into WKY rats. The density of β AR was significantly reduced as early as 3 days after the start of injection, and this receptor down-regulation of β AR has been reported by several laboratories^{18–20} Since most of these studies were conducted using isoproterenol as a stimulant it is difficult to extrapolate from those results to the effect of an enhanced sympathetic nerve activity as would occur in CHF. Unlike our results, Vatner et al reported an increase of β AR density after 4 weeks NE injection ($0.5 \mu\text{g/kg/min}$) in dogs²¹ Since we used nearly the same dose of NE and the same procedure (implanted osmotic pump) as those investigators, the reason for the differences in β AR regulation would likely reflect species difference. Chronic NE infusion also increased expression of G_i , but this response required more than 3 days infusion of NE (Fig. 4). This indicates that the overexpression of G_i required a more persistent stimulation of adrenergic receptor than did the down-regulation of β AR. NE-induced β AR down-regulation and overexpression of G_i have been also reported in in vitro studies with cardiac myocytes²²

In this study, 2 different animal models were investigated: BIO53.58, a genetic model for CHF, and NE-infused rats, a model which simulates the neurohumoral milieu with respect to increased NE in failing human hearts. Alterations observed in each

component of the adenylyl cyclase system (a decrease of β AR density, increased amount of G_i and decrease in catalytic activity) were quite similar in the 2 models, suggesting that alterations in failing hearts might be secondary phenomena provoked by elevated plasma catecholamine concentrations. Moreover, there was a chronological order in development of the changes in adenylyl cyclase system: a rapidly responding component (β AR) and slowly responding components (G_i and catalytic protein). In view of the time scale of response, the changes in post-receptor components (especially overexpression of G_i) might be a consequence of catecholamine-induced gene regulation. In fact, Easchenhagen et al recently observed an isoprenaline-induced increase in $G_{i2} \alpha$ mRNA in rat hearts²³ One interpretation of the changes that occur in CHF is that they are part of compensatory reactions that protect the intracellular environment from β AR over stimulation by reducing signal transducing efficiency through the adenylyl cyclase system. At the same time, however, these changes are somewhat counterproductive because the failing hearts mainly depend on adrenergic support to overcome its impaired pump function.

REFERENCES

1. CLARK RB: Advance in Cyclic Nucleotide and Protein Phosphorylation Research 20 (GREEN-GARD P, ROBINSON GA eds.): New York, Raven Press, 1986; 151–160
2. BRISTOW MR, GINSBURG R, MINOBE W, CUBICCIOTTI RS, SAGEMAN WS, LURIE K, BILLINGHAM ME, HARRISON DC, STINSON EB: *N Engl J Med* 1982; **307**: 205–211
3. DENNIS AR, MARSH JD, QUIGG RJ, GORDON JB, COLUCCI WS: *Circulation* 1989; **79**: 1028–1034
4. SCHWINGER RH, BÖHM M, ERDMANN E: *J Cardiovasc Pharmacol* 1990; **15**: 692–697
5. GILSON N, BOUANANI NEH, CORSIN A, CROZATIER B: *Am J Physiol* 1990; **258** (Heart Circ Physiol 27): H634–H641
6. BRODDE OE: *Pharmacol Rev* 1991; **43**: 203–242
7. FELDMAN AM, CATES AE, VEAZEY WB, HERSHBERGER RE, BRISTOW MR, BAUGHMAN KL, BAUMGARTNER WA, VAN DOP C: *J Clin Invest* 1988; **82**: 189–197
8. BÖHM M, GIERSECHIK P, JAKOBS KH, SCHNABEL P, KEMKES B, ERDMANN E: *Am J Cardiol* 1989; **64**: 812–814
9. URASAWA K, IGARASHI Y, KAWAGUCHI H, YASUDA H: *Jpn Circ J* 1990; **54**: 980–981
10. HARDING SE, JONES SM, O'GARA P, VES-

- COVO G, POOLE-WILSON PA: *Am J Physiol* 1990; **259** (Heart Circ Physiol): H1009–H1014
11. MURAKAMI T, KATADA T, YASUDA H: *J Mol Cell Cardiol* 1987; **19**: 199–208
12. KATADA T, OINIMA M, UI M: *J Biol Chem* 1986; **261**: 8182–8188
13. SALOMON Y, LONDOS C: *Anal Biochem* 1974; **58**: 541–548
14. SCHAFFNER W, WEISSMAN C: *Anal Biochem* 1973; **56**: 502–514
15. HOMBURGER F: *Ann N Y Acad Sci* 1979; **317**: 2–17
16. BÖHM M, GIERSEHIK P, JAKOBS KH, PIESKE B, SCHNABEL P, UNGERER M, ERDMANN E: *Circulation* 1990; **82**: 1249–1265
17. FOWLER MB, LASER JA, HOPKINS GL, MINOBE W, BRISTOW MR: *Circulation* 1986; **74**: 1290–1301
18. TSE J, POWELL JR, BASTE CA, PRIEST RE, KUO JF: *Endocrinology* 1979; **105**: 246–255
19. CHANG HY, KLEIN RM, KUNOS G: *J Pharmacol Exp Ther* 1982; **221**: 784–789
20. MAISEL AS, ZIEGLER MG, CARTER S, INSEL PA, MOTULSKY HJ: *J Clin Invest* 1988; **82**: 2038–2044
21. VATNER DE, VATNER SF, NEJIMA J, UEMURA N, SUSANNI EE, HINTZE TH, HOMCY CJ: *J Clin Invest* 1989; **84**: 1741–1748
22. REITHMANN C, GIERSEHIK P, SIDIROPOULOS D, WERDAN K, JAKOBS KH: *Eur J Pharmacol* 1989; **172**: 211–221
23. ESCHENHAGEN T, MENDE U, NOSE M, SCHMITZ W, SCHOLZ H, WARNHOLTZ A, WÜSTEL JM: *Naunyn-Schmiedeberg's Arch Pharmacol* 1991; **343**: 609–615