Measurement of Adenylate Cyclase Activity in the Right Ventricular Endomyocardial Biopsy Samples From Patients With Chronic Congestive Heart Failure

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A highly sensitive fluorometric assay technique was adopted in order to examine the adenylate cyclase activity in the minute right ventricular endomyocardial biopsy samples from patients with chronic congestive heart failure ($n = 10$). Norepinephrine (10^{-4} M) and adenosine (10^{-3} M) were incubated for 30 min with 10 μ l of membrane preparation (1–2 mg protein/mg) to analyze the extent of the receptor-coupled adenylate cyclase activity. Forskolin (10^{-4} M) stimulation was used to estimate the maximum adenylate cyclase activity (pmol/mg protein/min, mean $\pm$ SE). The new microanalytical cyclic AMP assay involves four steps: enzymatic destruction of noncyclic adenine nucleotides and phosphorylated metabolites, conversion of cyclic AMP to ATP, amplification of ATP by enzymatic cycling, and fluorometric measurement of

NADPH, which is generated in proportion to initial cyclic AMP levels. Basal and forskolin-stimulated maximum adenylate cyclase activities were 75 ± 8 and 123 ± 15 , respectively. Norepinephrine increased the adenylate cyclase activity to 107 ± 14 , while adenosine tended to decrease it to 65 ± 7 . In addition, elimination of adenosine by adenosine deaminase (10 U/ml) slightly increased the adenylate cyclase activity to 82 ± 9 . These results indicate that the adenylate cyclase activity can be measured in minute endomyocardial biopsy samples. Use of this new approach shows promise of becoming a new and potentially important way to predict the efficacy of pharmacological treatment. *J. Clin. Lab. Anal.* 14:48–52, 2000. © 2000 Wiley-Liss, Inc.

Key words: congestive heart failure; enzymatic fluorometric assay; adenosine; forskolin; norepinephrine; cyclic AMP

INTRODUCTION

Adenylate cyclase is a pivotal enzyme involved in adrenergic, cholinergic, and purinergic signal transduction of the heart. Although essential to the pharmacological control of inotropic function of patients with chronic congestive heart failure, the effect of drugs on the adenylate cyclase activity has not been examined as a diagnostic and therapeutic tool. While several investigators have measured the adenylate cyclase activity in endomyocardial biopsy specimen by using radioimmunoassay or the Salomon method (1–6), it is still difficult to systematically estimate the effect of drugs on the adenylate cyclase due to the limited amount of biopsy samples and the lack of a versatile yet sensitive assay method for adenylate cyclase activity.

To begin to overcome this methodological barrier, we adopted a recently developed, highly sensitive, nonradioactive enzymatic fluorometric assay technique (7–10), and ex-

amined the effects of pharmacological interventions on the adenylate cyclase activities in minute membrane preparation made from human right ventricular biopsy samples. In this study, norepinephrine, adenosine, and forskolin were incubated with the membrane preparation to analyze the extent of the receptor-coupled influences on the adenylate cyclase activity.

MATERIALS AND METHODS

Informed consent was obtained from each patient involved in the present study. All experimentation was performed ac-

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Production of the Plasma Membrane Preparation

Biopsy specimens were obtained by cardiac catheterization from the right endomyocardial surface of the interventricular septum. Three specimens were taken from each patient with chronic congestive heart failure. Two samples were used for histological evaluation. One biopsy sample, weighing approximately 1 mg, was immediately frozen and stored at -80°C for the measurement of adenylate cyclase activity. This sample was placed in the microcentrifugation tube and homogenized with 500 μL of ice-cold SET buffer (0.25 M sucrose; 0.1 mM EDTA; 5.0 mM Tris-HCl) using a pellet mixer. The mixture was centrifuged at $10,000g$ for 5 min at 4°C . The pellet was resuspended in the 500 μL of SET buffer and the mixture was centrifuged under the same setting. After this procedure was repeated twice, the pellet was finally suspended in 100 μL of SET buffer. Protein analysis was performed using a commercially available protein assay reagent (Pierce, Rockford, IL). The membrane suspension was diluted with SET buffer to a concentration of 1–2 mg protein /ml, and it was stored at -80°C until its adenylate cyclase activity was measured.

Enzymatic Fluorometric Assay of Adenylate Cyclase Activity

The adenylate cyclase activity of the membrane preparation was measured with an enzymatic fluorometric assay technique that was essentially the same as those previously described (7–10). Each reaction is summarized in Fig. 1. The assay consists of two parts; namely, the production of cyclic AMP by the adenylate cyclase in the membrane preparation (part A), and the measurement of newly formed cyclic AMP (part B).

Part A (production of cyclic AMP)

Ten μL of adenylate cyclase mix (100 mM Tris-acetate, pH 7.4; 20 mM KCl; 10 mM MgCl_2 ; 20 mM phosphoenolpyruvate; 2 mM ATP; 20 μM GTP; 2 mM dithiothreitol; 0.04% bovine serum albumin; 0.1 mM 3-isobutyl-1-methylxanthine [IBMX]; 100 $\mu\text{g}/\text{mL}$ pyruvate kinase) was added to each microcentrifugation tube in duplicate with or without either of adenosine (2×10^{-3} M), adenosine deaminase (20 U/ml), forskolin (2×10^{-4} M) or norepinephrine (2×10^{-4} M) as previously described (7–10). Next, the membrane suspension in a volume of 10 μL was added to each tube. The reaction mixture and membrane suspension, both before and after being mixed, were maintained at 4°C to ensure the same starting time for all assay tubes. The reaction was initiated by placing

Fig. 1. Diagram of reactions in the enzyme fluorometric assay for adenylate cyclase activity. Part A, production of cyclic AMP by the adenylate cyclase in the membrane preparation; Part B, the new microanalytical cyclic AMP assay. Enzymatic destruction of noncyclic adenine nucleotides and

phosphorylated metabolites (step 1). Conversion of cyclic AMP to ATP (step 2). Amplification of ATP by enzymatic cycling (step 3). Fluorometric measurement of NADPH, which is generated in proportion to initial cyclic AMP levels (step 4). P_i , inorganic phosphate.

the tubes in a water bath maintained at 37°C. After 30 min, the reaction was terminated by heating at 90°C for 3 min. The mixture was vortexed 3 times and centrifuged at 10,000g for 5 min. The supernatant solution was stored for the following cyclic AMP assay.

Part B (enzymatic fluorometric measurement of cyclic AMP)

A volume of 5 μ L of the supernatant was transferred to a 10 $\times$ 75 mm disposable assay tube (Iwaki Lab Ware, Tokyo, Japan) in triplicate. For the cyclic AMP standard, 5 μ L of a known amount of cyclic AMP was added to the tubes. The cyclic AMP assay consisted of the following 4 steps: (1) enzymatic destruction of noncyclic adenine nucleotides and phosphorylated metabolites: a volume of 25 μ L of reaction mix A (100 mM Tris-HCl, pH 8.0; 2 mM MgCl₂; 4 units/ml apyrase; 10 units/ml adenosine deaminase; 40 units/ml alkaline phosphatase) was added to each tube. The mixture was incubated at 37°C for 10 min to destroy potential interfering substances including noncyclic adenine nucleotides and phosphorylated metabolites. Enzymes were then destroyed by heating for 3 min at 95°C; (2) conversion of cyclic AMP to ATP: a volume of 25 μ L of reaction mix B (100 mM Tris-HCl, pH 8.0; 2 mM MgCl₂; 0.01% bovine serum albumin; 150 mM KCl; 2 mM dithiothreitol; 40 nM ATP; 8 mM phosphoenolpyruvate; 12 units/ml phosphodiesterase; 4.5 units/ml myokinase; 80 units/ml pyruvatekinase) was added to each tube from step 1. The mixture was incubated for at least 6 hr at room temperature to completely convert cyclic AMP to ATP. This was usually performed by an overnight incubation; (3) enzymatic amplification of ATP to fructose-6-phosphate: a volume of 25 μ L of reaction mix C (100 mM Tris-HCl, pH 8.0; 2 mM MgCl₂; 0.01% bovine serum albumin; 8 mM fructose; 60 units/ml hexokinase) was added to the tubes from step 2 on ice. The mixture was incubated for 1 hr at 37°C to amplify ATP to about 6,000 times of fructose-6-phosphate; (4) conversion of fructose-6-phosphate to NADPH: a volume of 125 μ L of reaction mix D (100 mM Tris-HCl, pH 8.0; 3.2 mM EDTA; 0.6 mM NADP⁺, 1 unit/ml phosphoglucose isomerase; 0.5 unit/ml glucose-6-phosphate dehydrogenase) was added to the tubes from step 3. After the mixture was incubated for 10 min at room temperature, a volume of 600 μ L of distilled water was added to each tube. The concentration of NADPH, formed when the glucose-6-phosphate was converted to 6-phosphogluconolactone, was measured as an emission fluorescence of 460 nm by the 340 nm of excitation wave using fluorometric spectrophotometer (Hitachi, F3000, Tokyo, Japan). Total assay duration was about 10–12 hr, including an overnight incubation step.

Biochemicals

Apyrase, phosphoenolpyruvate, phosphodiesterase, IBMX, sucrose, fructose, dithiothreitol, norepinephrine, and forskolin

were obtained from Sigma Chemical Company (St. Louis, MO). All other enzymes and substrates were obtained from Boehringer-Mannheim Company (Indianapolis, IN).

Statistics

All data are presented as mean $\pm$ SE. The statistical comparisons of mean values between the pharmacological interventions were evaluated by one-way repeated-measures ANOVA followed by Contrasts for mean values comparison, whereas those between the subgroups classified by the pathological diagnosis were assessed by one-way factorial ANOVA. The *P* values < 0.05 were considered significant.

RESULTS

Biopsy samples were obtained from 9 men and 1 woman with an average age of 53 ± 2 years. A summary of the background data of each patient is shown in Table 1. Most of the drugs were withheld at least five half-lives before the catheterization except the drugs shown in Table 1. Each sample was assayed in triplicate. The difference of the assay values for each sample varied < 10%. The longest storage time was 12 months and that did not alter the basal or stimulated adenylate cyclase activity. For each assay, the amount of tissue was enough to measure basal adenylate cyclase activity and adenylate cyclase activity after stimulation with up to five different agonists.

The effects of β -adrenergic or forskolin stimulation on adenylate cyclase activity in membrane preparations obtained from the human samples are summarized in Figure 2. Basal and forskolin (10^{-4} M)-stimulated adenylate cyclase activities (cyclic AMP pmol/min/mg protein, *n* = 10) were 75 ± 8 and 123 ± 15 , respectively. A significant difference was detected between these two values. Norepinephrine (10^{-4} M) also significantly increased the adenylate cyclase activity to 107 ± 14 (cyclic AMP pmol/min/mg protein, *n* = 10). The increases of the adenylate cyclase activity over the basal values by forskolin and norepinephrine were $+65 \pm 11$ (%) and $+43 \pm 10$ (%), respectively.

TABLE 1. Background data of the patients^a

Patient No.	Age	Sex	Diagnosis	Drugs not withheld
1	50	M	DCM	Furosemide
2	44	M	DCM	Furosemide, digoxin
3	60	M	DCM	Furosemide
4	42	M	DCM	None
5	54	F	HCM	None
6	55	M	HCM	None
7	57	M	HCM	None
8	60	M	HHD	Furosemide, isosorbide dinitrate
9	53	M	HHD	None
10	51	M	HHD	Furosemide

^aDCM, dilated cardiomyopathy; HCM, hypertrophic cardiomyopathy; HHD, hypertensive heart disease.

Fig. 2. Effects of adenosine (10^{-3} M), adenosine deaminase (10 U/ml), norepinephrine (10^{-4} M), and forskolin (10^{-4} M) on adenylate cyclase activity of the minute membrane preparation made from a biopsy sample of human heart. Experiments were performed in ten different patients with chronic congestive heart failure. The number in each symbol indicates the patient number shown in Table 1. Data are expressed as mean $\pm$ SE. ADA, adenosine deaminase; NE, norepinephrine; DCM, dilated cardiomyopathy; HCM, hypertrophic cardiomyopathy; HHD, hypertensive heart disease. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

As summarized in Figure 2, adenosine (10^{-3} M) tended to decrease the adenylate cyclase activity to 65 ± 7 (cyclic AMP pmol/min/mg protein, $n = 10$), which was -14 ± 3 (%) from the basal adenylate cyclase activity, but this change was not statistically significant. We also measured the adenylate cyclase activity in the presence of adenosine deaminase (10 U/ml), which completely converted adenosine into inosine and NH_3 . Elimination of adenosine by adenosine deaminase slightly increased the basal adenylate cyclase activity to 82 ± 9 (cyclic AMP pmol/min/mg protein, $n = 10$), which was $+9 \pm 2$ (%) over the basal adenylate cyclase activity. There was no statistically significant difference in the activities between the basal and adenosine deaminase treated preparations; however a significant difference was detected in the activities between the adenosine- and adenosine deaminase-treated preparations.

Since the pathophysiological changes in the heart of each patient may affect the current results, we performed a subgroup analysis of the adenylate cyclase activities in each pharmacological intervention. The patients were divided into three groups based on their pathological diagnosis: namely, dilated cardiomyopathy group ($n = 4$), hypertrophic cardiomyopathy group ($n = 3$) and hypertensive heart disease group ($n = 3$). The adenylate cyclase activities of each patient are also plotted on the Figure 2. Although the number of patients is low, the adenylate cyclase activities in the hypertensive heart disease group tended to be greater than in other groups.

DISCUSSION

The results from this study demonstrate that it is feasible with the new fluorometric method to measure adenylate cyclase activity in a single endomyocardial biopsy sample. While others have used radioimmunoassay and the Salomon method to measure adenylate cyclase activity, these are not sensitive or flexible enough to measure adenylate cyclase activity in a small amount of tissue as currently obtained from biopsy samples of the heart (7–12). Measurement of adenylate cyclase activity from samples weighing < 1 mg with the new approach may provide new insights into individualizing patient care.

The results from this study also demonstrate that while basal adenylate cyclase activities were similar to those reported in the previous studies using larger tissue samples (1–6), the extent of the stimulation by norepinephrine as well as forskolin was not so great (1,4,5,9,10). The most straightforward explanation for this attenuation of adenylate cyclase stimulation by the β -receptor pathway is down regulation of the receptor-effector pathway. Because the cardiac tissue obtained using the current biopsy technique is from the endocardial layer, subcellular remodeling of the endocardium due to functional stress of congestive heart failure might be related to our present observation. An alternative explanation is the inhibitory effect of adenosine. Because adenosine was not removed during the assay examining the effects of norepinephrine and forskolin, the stimulatory effects may be offset by endogenous adenosine which might tonically inhibit adenylate cyclase (1,13).

In summary, the enzymatic fluorometric assay provides a sensitive, relatively rapid, and inexpensive method for measuring the adenylate cyclase activity in minute biopsy samples of human heart. While experiments are being conducted to optimize the assay conditions for each pharmacological treatment, the current method may become a new and potentially important way to predict the efficacy of pharmacological treatment.

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