Pertussis Toxin Treatment of Whole Blood
A Novel Approach to Assess G Protein Function in Congestive Heart Failure

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This study was designed to assess G protein function in mononuclear leukocytes (MNL) of patients with congestive heart failure (CHF). MNL membranes were ADP-ribosylated in vitro in the presence of pertussis or cholera toxin. The amount of pertussis toxin substrates did not differ significantly between CHF patients (6,100±224 fmol/mg, n=23) and age-matched healthy control subjects (5,812±972 fmol/mg protein, n=19). Among the CHF patients, no differences were observed between those with idiopathic and ischemic CHF. The amount of cholera toxin substrates also did not differ significantly between CHF patients (7,522±1,405 fmol/mg protein, n=11) and control subjects (5,654±707 fmol/mg protein, n=14). Moreover, basal and isoproterenol- and prostaglandin E₁-stimulated cyclic AMP (cAMP) accumulation in MNL was similar in control subjects and patients. To detect more subtle alterations of the cAMP-generating system, we incubated anticoagulated blood with 250–400 ng/ml pertussis toxin for 4 hours at 37°C. This treatment completely ADP-ribosylated the MNL pertussis toxin substrates. Incubation with pertussis toxin did not change basal or prostaglandin E₁-stimulated cAMP generation in MNL of control subjects, but it significantly enhanced stimulated generation (443±44 vs. 643±93 pmol/10⁷ cells, p<0.025) in MNL of CHF patients. This enhancement was most pronounced in the most severely ill patients (New York Heart Association class IV) and correlated with plasma norepinephrine levels, another marker of CHF severity (r=0.798, n=11, p<0.01). Isoproterenol-stimulated cAMP generation, which was much weaker than that produced by prostaglandin E₁, was enhanced only in patients in class IV. We conclude that despite apparently unaltered amounts of subunits identifiable by ADP ribosylation with pertussis toxin, MNL of CHF patients appear to have enhanced functional activity (i.e., inhibition of cAMP formation) by these substrates. Thus, incubation of MNL with pertussis toxin reveals a tonic inhibition of cAMP generation in cells of CHF patients. The results suggest that incubation of whole blood with pertussis toxin and subsequent assessment of cAMP accumulation in MNL provide a useful way to examine G protein function in patients with cardiovascular disease. (Circulation 1990;81:1198–1204)

Inotropic responsiveness is markedly reduced in the hearts of experimental animals and patients suffering from congestive heart failure (CHF). A reduced density of receptors for catecholamines (β-adrenergic) and vasoactive intestinal polypeptide might partly explain this reduction in inotropy.¹⁻⁴ An increasing body of evidence suggests that alterations of the signal transduction pathways by which these receptors activate cellular responses also contribute to the impairment of cardiac contractility. Specifically, alterations of cardiac GTP-binding (G) proteins have been demonstrated in CHF,⁵⁻¹⁰ but the published data are unsatisfactory in several ways. 1) It is not clear whether the number or activity of G proteins is altered. 2) Previous data in human hearts were exclusively obtained from patients with end-stage CHF undergoing cardiac transplantation; such data do not allow conclusions on the role of G

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protein alterations during the development of CHF. 3) The possibility that alterations of signal transduction occur in tissues other than the heart has not been extensively tested in CHF.

Therefore, we studied signal transduction in an extracardiac tissue, in circulating mononuclear cells (MNL), in patients with different stages of CHF, and in age-matched healthy control subjects. For this purpose, we developed a novel assay that involves treatment of intact cells with pertussis toxin and subsequent assessment of cyclic AMP (cAMP) accumulation. Pertussis toxin catalyzes the transfer of an ADP-ribose moiety from NAD to the α-subunit of a variety of G proteins and thereby can inhibit the activity of these proteins. One of these pertussis toxin–sensitive G proteins is Gs so named because it inhibits the activity of adenyl cyclase. In this study, we show that patients with CHF appear to have enhanced activity of Gs.

Methods

Materials

[^32P]NAD and [3H]cAMP were obtained from New England Nuclear (Boston, Massachusetts). Pertussis and cholera toxins were obtained from List Laboratories (Campbell, California). All other reagents were from standard sources and of the highest commercially available purity grade.

Measurement of Pertussis and Cholera Toxin Substrates

Subjects. Twenty-three male patients from San Diego Veterans Administration Medical Center (age range, 44–83 years; mean, 65±2 years) with chronic CHF (New York Heart Association [NYHA] classes II–IV) were studied, 11 with idiopathic (mean age, 64±3 years) and 12 with ischemic cardiomyopathy (mean age, 65±2 years). The left ventricular ejection fraction, determined by equilibrium-gated radionuclide ventriculography, averaged 34±3% for patients with idiopathic cardiomyopathy and 32±4% for patients with ischemic cardiomyopathy. All patients were receiving digitalis, diuretics, and at least one vasodilator (nitrates in 16, hydralazine in 12, and angiotensin converting enzyme inhibitors in 13 subjects). All cardiac medications were withheld for 12–24 hours before the study. Nineteen men of similar age (range, 35–92 years; mean, 64±4 years) with no history of cardiac impairment served as control subjects.

Protocol. Subjects reported in a fasting state to the cardiology department, and each had an indwelling catheter placed in a forearm vein. They then lay supine in a quiet room for 20 minutes, at which time a venous blood sample was drawn (100 ml).

Preparation of MNL. MNL were isolated and washed at 4°C according to previously published techniques. The MNL were washed twice in phosphate-buffered saline solution, and then washed and resuspended in Dulbecco’s modified Eagle’s medium supplemented with 20 mM HEPES and 1 mg/ml bovine serum albumin (pH 7.4). Between 85% and 95% of these cells were lymphocytes; the rest were monocytes.

To prepare membranes, the MNL were resuspended at 5×10^7 cells/ml and spun for 1 minute at 14,000g in a microfuge (IEC, Mountain View, California), and the pellet was freeze/thawed using liquid nitrogen and a water bath. The pellet was resuspended with 1 ml lysing buffer (10 mM Tris, 2 mM MgCl₂, and 100 μM EGTA), freeze/thawed three times, and spun exactly 5 seconds in a microfuge. The supernatant was spun at 12,500g for 10 minutes while cold, and the pellet was brought up to the desired volume with 20 mM Tris, 1 mM EDTA, and 1 mM dithiothreitol (DTT), pH 8.0.

[^32P]ADP ribosylation of Gᵣ and Gₛ. [32P]ADP ribose incorporation into Gₛ in the presence of pertussis toxin with [32P]NAD substrate (specific activity, 30–40 Ci/mmol) was modified from the method of Bokoch et al. Ten microliters of MNL membranes (about 10 μg protein) were incubated in 28 μl of a solution containing 10 mM DTT, 100 mM Tris, 10 mM thymidine, 100 μM GTP, 1.0 mM ATP, 2.5 mM MgCl₂, 1.0 mM EDTA, 500 μM B-NADP, 1 μM [32P]NAD, and 0.2 μg pertussis toxin at pH 8.0 for 60 minutes at 30°C. Preactivation of pertussis toxin was not necessary to achieve maximal ribosylation if the toxin was added simultaneously with 10 mM DTT. In preliminary experiments involving time course studies and different amounts of protein and of NAD, we determined that ADP ribosylation under these conditions was maximal and that detergent extraction by cholate was not required for maximum labeling. Reincubation of the membranes with fresh toxin or fresh NAD did not improve the extent of ADP ribosylation.

ADP ribose incorporation into Gₛ in the presence of cholera toxin was modified from the method of Longabaugh et al. Cholera toxin was activated by incubation with 20 mM DTT for 30 minutes at 30°C. Activated cholera toxin was incubated with MNL membranes (about 20 μg protein), 125 mM KHPO₄, 20 mM phosphocreatine, 10 mM thymidine, 60 U/ml creatine phosphokinase, 5 mM ATP, 250 μM GTP, 1.25 mM NADP, 5 mM MgCl₂, 10 μM NAD, and 5 μM [32P]NAD for 90 minutes at 30°C. Time course and concentration-response experiments for NAD and cholera toxin were performed initially to ensure that ribosylation was maximal under these conditions. We also tested whether addition of exogenous ADP ribosylation factor was necessary for maximal ribosylation of Gₛ. For this purpose, we prepared membranes from cyc S49 cells (which lack Gₛ) and performed the ribosylation experiments in the presence and absence of these membranes. Our experiments showed that ADP ribosylation factor was not necessary to achieve maximal ribosylation in control subjects or in CHF patients (data not shown). Furthermore, detergent extraction of the membrane tissue was not required for maximal labeling.
The reaction for labeling of both Gα and Gβ was terminated by adding 38 μl gel sample buffer containing 125 mM Tris (pH 6.9), 20% glycerol, 4% sodium dodecyl sulfate, and 10% 2-mercaptoethanol and bromophenol blue. The samples were boiled for 5 minutes, loaded on slab gels (running gel 4% and stacking gel 17% acrylamide), and run overnight at a constant current of 20 mA. The gels were then stained with Coomassie blue, destained, and dried for 2 hours on filter paper with a gel dryer (Bio-Rad Labs, Richmond, California). Autoradiography of the dried gels was performed at −70°C for 3 days with Kodak X-omat XAR-5 film in the presence of a lightning-plus-intensifying screen. Molecular weight standards were run in each gel, and the autoradiograms and the respective original Coomassie-stained gel were aligned to identify the apparent molecular weight of the ADP-ribosylated proteins.

To determine the amount of [32P]ADP-ribose incorporated into the G proteins, individual bands were localized on the autoradiograph, cut from the lanes, and counted in 20 ml scintillation fluid. For analysis of Gα, the 39 and 41 kDa bands were cut out and pooled together. Each lane of a given gel was loaded with the same amount of protein (whether MNL were from CHF or from control subjects), and one lane of each gel was run in the absence of pertussis or cholera toxin but loaded with the same amount of protein (control lane). Specifically, incorporated counts (range, 500–1,500 cpm) were calculated by subtracting the counts in the control lane (approximately, 400 cpm) from those incorporated in the presence of pertussis or cholera toxin. The protein content of membrane preparations was quantified by the method of Lowry et al.15 Using the specific activity of the NAD and the amount of protein loaded to each lane, we calculated the ADP ribosylation and expressed it as femtomoles of [32P]ADP-ribose incorporated per milligram protein. Catecholamine determinations were performed by a radioenzymatic assay.16

Measurement of cAMP Formation in MNL After Pertussis Toxin Treatment

Patients. Thirteen male patients (age range, 56–73 years; mean, 59±5 years) with CHF (NYHA classes II–IV) were studied. Each patient had a clinical history of CHF for at least 6 months. Six patients were classified as having ischemia and seven as idiopathic cardiomyopathy. The left ventricular ejection fraction, determined by equilibrium-gated radioisotope ventriculography, averaged 25±3%. All patients were receiving digitalis, diuretics, and at least one vasodilator (nitrates in 12, hydralazine in two, and angiotensin converting enzyme inhibitors in seven patients). All cardiac medications were withheld for 12–24 hours before the study. Eight healthy men (age range, 32–76 years; mean, 61±5 years) who had no history of cardiac impairment served as control subjects.

Incubation of blood with pertussis toxin. Subjects reported to the cardiology laboratory as detailed above. Eighty milliliters of blood was obtained and placed in tubes containing EDTA (5 mM final) and incubated with or without pertussis toxin (250–400 ng/ml) for 4 hours at 37°C in an incubator with 5% CO2. The concentration of pertussis toxin needed to completely ribosylate the αλτπαλα-subunit of Gα in these intact cell studies varied depending on the age and lot number of pertussis toxin.

cAMP accumulation. MNL were prepared from the blood after the incubation with pertussis toxin as described above. cAMP accumulation in these MNL was then assessed by adding 0.1 ml cells (2–10×105, ice cold) to 0.9 ml Dulbecco’s modified Eagle’s medium supplemented with 20 mM HEPES and 1 mg/ml bovine serum albumin at 37°C. The final reaction mixture contained 100 μM isobutylmethylxanthine and 100 μM Ro 20-1724 (to inhibit cyclic nucleotide phosphodiesterase), 10 μg/ml each of superoxide dismutase and catalase (to prevent oxidation of the isoproterenol17), and in some tubes 10 μM isoproterenol or 10 μM prostaglandin E1. The reactions were terminated after 2 minutes by centrifuging at 10,000g for 20 seconds, aspirating the supernatant, resuspending the pellet in 100 μl of 50 μM sodium acetate (pH 4.0) that contained 0.2 mM isobutylmethylxanthine, and placing the tubes in a boiling water bath for 5 minutes. The tubes were then frozen, and aliquots were later assayed for cAMP with a competitive protein-binding method18 with previously described minor modifications.13,19 All assays were performed in quadruplicate, and the SEM of each quadruplicate was typically less than 10%.

Data evaluation. Unless otherwise stated all data in text, tables, and figures are mean±SEM of n experiments. Significance of differences was calculated by two-tailed t tests as appropriate.

Results

Pertussis Toxin–Catalyzed ADP Ribosylation

Pertussis toxin catalyzed the incorporation of [32P]ADP-ribose into two major bands with apparent molecular weights of 39 and 41 kDa, which is in good agreement with the previously described pertussis toxin substrates Gα and Gβ, respectively.11 The incorporation of radioactivity into these two bands increased linearly with protein content throughout a range of 2.5–10 μg membrane protein (Figure 1).

The amount of [32P] incorporated into lymphocyte membranes from patients with CHF (5,812±972 fmol/mg, n=23) did not differ significantly from that of age-matched controls (6,100±224 fmol/mg, n=19) (Figure 2). There were no significant differences between patients with idiopathic and ischemic CHF (Figure 2) or between patients with CHF grouped according to NYHA classification (data not shown).

Cholera Toxin–Catalyzed ADP Ribosylation

Cholera toxin catalyzed the ADP ribosylation by [32P]NAD into a major band with an apparent molecular weight of 43–45 kDa. [32P] incorporation into
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**FIGURE 1.** Relation between protein concentration and maximal pertussis toxin-mediated ADP ribosylation. Varying amounts of membranes from normal human lymphocytes were incubated with activated pertussis toxin for 60 minutes and then electrophoretically separated on sodium dodecyl sulfate–polyacrylamide gels. An autoradiogram of the 39–41 kDa ADP-ribosylated peptide is shown.

This band also increased linearly with protein content (data not shown). Figure 3 shows an example of cholera toxin-catalyzed incorporation of $^{32}$P into mononuclear leukocyte membranes from three patients: control, idiopathic cardiomyopathy, and ischemic cardiomyopathy.

**cAMP Accumulation in Intact MNL**

Basal, isoproterenol- and PGE$_1$-stimulated cAMP accumulation was similar in MNL from CHF patients and healthy control subjects (Figure 4). To investigate more subtle alterations of the cAMP generating system, we incubated whole blood with 250–400 ng/ml pertussis toxin for 4 hours at 37°C. This treatment of the intact cells resulted in a complete

**FIGURE 2.** Bar graph of pertussis toxin-catalyzed incorporation of $^{32}$P into mononuclear leukocyte membranes from patients with congestive heart failure (CHF: idiopathic and ischemic) and age-matched controls. Data are mean±SEM.

**FIGURE 3.** Autoradiogram depicting cholera toxin-catalyzed incorporation of $^{32}$P in mononuclear leukocyte membranes from three patients: control, idiopathic cardiomyopathy, and ischemic cardiomyopathy.

**FIGURE 4.** Bar graph of cyclic AMP accumulation in mononuclear leukocytes from normal control subjects (n=9) and patients with congestive heart failure (n=13). cAMP was measured in pM/10$^7$ cells under basal conditions and after stimulation with 10 µm isoproterenol (ISO) or 10 µM prostaglandin E$_1$ (PGE$_1$). Data are mean±SEM.
ADP ribosylation of the MNL pertussis toxin substrates, because membranes prepared from these MNL did not specifically incorporate any $^{32}$P into bands with apparent molecular weights of 39 or 41 kDa on incubation with fresh pertussis toxin and $^{32}$P-NAD (data not shown).

The basal cAMP accumulation in MNL was not significantly different between pertussis toxin-treated and untreated cells in control subjects and CHF patients (Figure 5). The isoproterenol- and PGE$_1$-stimulated cAMP accumulation in MNL from healthy control subjects was not significantly different between pertussis toxin-treated and untreated cells (Figure 6). In contrast, incubation with pertussis toxin significantly increased PGE$_1$-stimulated cAMP accumulation in MNL from patients with CHF (from $443\pm44$ to $643\pm93$ pmol/10$^7$ cells; $n=15$, $p<0.025$, Figure 6). Incubation of MNL with pertussis toxin did not significantly increase isoproterenol-stimulated cAMP accumulation in MNL from patients with CHF (from $234\pm35$ to $254\pm62$ pmol/10$^7$ cells, Figure 6). We did not observe any relation between basal or stimulated cAMP accumulation and age in normal subjects or patients with CHF either before or after pertussis toxin treatment (data not shown).

We used two approaches to determine whether the alteration of cAMP accumulation after pertussis toxin treatment were related to the severity of CHF. First, each of the four CHF patients in NYHA class IV had greater cAMP accumulation in response to isoproterenol and PGE$_1$ in MNL incubated with pertussis toxin ($p=0.29$ and $p=0.03$ by paired t test, respectively, data not shown). Second, because plasma norepinephrine levels have been used as a prognostic indicator in heart failure, we also subdivided our patients according to this parameter. Isoproterenol-stimulated cAMP generation after pertussis toxin treatment was significantly elevated only in the group with plasma norepinephrine values greater than 700 pg/ml but not in the groups with lower plasma catecholamine levels ($p=0.02$, Figure 7). PGE$_1$-stimulated cAMP generation was enhanced after pertussis toxin treatment in all three groups ($p=0.02$), and the enhancement was greatest at higher plasma catecholamine levels (Figure 7). This finding was further documented by comparing plasma norepinephrine levels and the enhancement of PGE$_1$-stimulated cAMP accumulation after pertussis toxin treatment for each patient. This comparison yielded a significantly positive correlation ($r=0.798$, $n=11$, $p<0.01$).

Discussion
cAMP is an important mediator of inotropic effects in the human heart. A cAMP is formed by the catalytic unit of adenyl cyclase that is under the control of two homologous G proteins that mediate the stimulation (G$_i$) and inhibition (G$_s$) of the enzyme. A frequently used method for quantifying G$_i$ and G$_s$ involves ADP ribosylation of these two proteins in vitro as catalyzed by cholera and pertussis toxins, respectively. Determination of G$_i$ and G$_s$ in failing hearts by use of ADP ribosylation techniques has yielded conflicting results. Longabaugh et al$^5$ demonstrated a decrease of cholera toxin substrates (G$_s$) in hearts of dogs with heart failure produced by aortic banding. In contrast, three studies in humans have
reported increases in pertussis toxin substrates (Gₙ) in the hearts of patients with idiopathic cardiomyopathy with no change in cholera toxin substrates. In patients suffering from ischemic cardiomyopathy, pertussis toxin substrates were reported to decrease. Using antibody techniques, Ransnas et al. found a small reduction of Gₙ in a small number of patients with idiopathic CHF. In an animal model of idiopathic heart failure, the cardiomyopathic hamster, Gₙ activity appears to be reduced, because the activation of adenyl cyclase by NaF, forskolin, and isoproterenol is decreased and because muscle membranes from these animals show less Gₙ activity when reconstituted in cyc⁻ S49 cells (that lack Gₙ) than those from control hamsters. However, the amounts of Gₙ (as quantified by Western blot analysis), of mRNA for Gₙm and of pertussis toxin substrates are not reduced in the hearts of these hamsters. Thus, most findings suggest that the ratio of Gₙ to G₉ is decreased in failing hearts, but it cannot yet be determined whether numerical and functional alterations of Gₙ or G₉ are involved. This controversy partly stems from an uncertainty whether ADP ribosylation is an assay for the number or for the functional state of G proteins.

We have developed a new, simple method to assess the functional state of pertussis toxin substrates in cells from patients. To eliminate a possible inhibitory effect of Gₙ-like proteins on cAMP generation, we treated intact MNL with pertussis toxin. This treatment completely ribosylated pertussis toxin substrates in MNL, because no additional ADP ribosylation could be detected by further addition of fresh pertussis toxin to MNL membranes. Because treatment of MNL with pertussis toxin did not alter the basal or isoproterenol- and PGE₁-stimulated cAMP generation in MNL from normal subjects, pertussis toxin substrates do not appear to exert a major tonic inhibitory effect on adenyl cyclase in the MNL of healthy people. Basal and isoproterenol-stimulated cAMP accumulations, which are small, were also not significantly altered by pertussis toxin treatment in the group of patients with CHF. PGE₁-stimulated cAMP generation, however, which was considerably larger even in the absence of treatment with pertussis toxin, was significantly increased after pertussis toxin treatment in patients with CHF. These data suggest that cAMP generation in MNL from in vitro patients with CHF is under a greater tonic inhibitory influence of Gₙ than are cells from normal subjects.

Our method appears to have several advantages compared with other approaches. First, pertussis toxin treatment of intact MNL revealed inhibitory effects of Gₙ-like proteins that were not detected by pertussis or cholera toxin–catalyzed ADP ribosylation. Thus, our data suggest that pertussis toxin (Gₙ) substrates can undergo functional alterations in MNL that are not accompanied by major quantitative changes or pertussis toxin or cholera toxin substrates. Second, studies of circulating MNL can be complicated by a heterogeneity of lymphocyte subsets with regard to receptor number and responsiveness. As our technique uses the same cells treated with and without toxin, less complications from this should be expected. Third, our technique is simple, rapid, uses an easily accessible tissue, and requires use of less radioactivity than in ADP ribosylation studies.

Because MNL are an extracardiac tissue, their usefulness may be limited as a model to study possible alterations in the myocardium. The possible pitfalls of using MNL for monitoring cardiac β-adrenergic receptors were recently discussed. The interpretation of ADP ribosylation assays of circulating lymphocytes is also complicated because circulating MNL are composed of different subsets that vary in their cAMP responsiveness to various agents and thus probably have quantitatively different cAMP generating systems. This is important because numerous acute and chronic conditions including CHF can alter the subset composition of circulating lymphocytes. Our method of assaying the effect of pertussis toxin treatment of intact cells on cAMP generation is much less sensitive to alterations of the subset composition of circulating MNL, because the same cells are used in toxin-treated and control experiments. More positively, our data point to the intriguing possibility that coupling of receptors to adenyl cyclase in CHF patients might be altered not only in the myocardium but also in other tissues. Several lines of evidence support this speculation. 1) We have found that after dynamic exercise cAMP generation in response to isoproterenol and to PGE₁ is elevated in MNL of healthy controls but blunted in MNL of CHF patients. 2) A marked reduction of cholera toxin substrates in the MNL of CHF patients was recently reported by Horn et al.; the reason for the discrepancy between these findings and the present data is not clear and might be related to methodological differences in the ribosylation protocols. 3) The activity of Gₙ in the cardiomyopathic hamster is decreased not only in the myocardium but also in skeletal muscle. Thus, an alteration of receptor signal transduction system in extracardiac tissues of CHF patients appears to be likely. The pathophysiological consequences of such alterations remain to be assessed.

The availability of an easily accessible tissue, the MNL, for studies of G protein function in CHF should prove useful in various ways. Importantly, it would allow the monitoring of G protein function in patients during the course of development of CHF. We have made a preliminary attempt to do so by subgrouping our patients according to their NYHA class or to their plasma catecholamine levels. Our data demonstrate that the enhancement of PGE₁-stimulated cAMP accumulation by pertussis toxin treatment of intact cells was most pronounced in the most severely ill patients whether stratified according to NYHA classification or to plasma catecholamine levels. Moreover, we found a significant positive correlation between plasma norepinephrine concentrations and enhancement of PGE₁-stimulated cAMP...
accumulation by pertussis toxin. Interestingly, in the four CHF patients in NYHA class IV, isoproterenol-stimulated cAMP accumulation was also increased after pertussis toxin treatment. Conceivably heterologous desensitization by enhanced β-adrenergic receptor stimulation and a resultant enhancement in G, activity could be the cause of the alteration of the MNL signal transduction.

In conclusion, our data demonstrate that the cAMP generating system of an extracardiac tissue can be altered in CHF, that is, pertussis toxin substrates appear to exert a tonic inhibitory effect on cAMP generation in circulating MNL. This tonic inhibition is not detected by toxin-catalyzed ADP ribosylation of cell membranes. We speculate that enhancement in G, protein function may contribute to the development of CHF in cardiac and extracardiac locations.

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