β 1 and β 2 Adrenoceptor Ligand and mRNA Expression in Dilated Cardiomyopathy

Christer SYLVÉN,*^a Gunilla MISCHA,^a Eva JANSSON,^a Peter SOTONYI,^b Liang-Xiong FU,^c Finn WAAGSTEIN,^c and Mikael BRÖNNEGÅRD^b

Karolinska Institute at the Departments of Medicine, Clinical Physiology and Pediatrics, Huddinge Hospital,^a Huddinge, Wallenberg Laboratory, Sahlgrenska Hospital,^c Gothenburg, Sweden and Semelweis Institute,^b Budapest, Hungary.
Received April 10, 1995; accepted June 8, 1995

β 1 and β 2 adrenoceptor ligand activity has been shown to be down-regulated in failing myocardium. It is the aim of this study to test the hypothesis that also mRNA levels are down-regulated in dilated cardiomyopathy.

β 1 and β 2 adrenoceptor ligand activities and mRNA expressions were analyzed in left ventricular biopsies from six organ donor hearts, in papillary muscles from seven patients operated on for mitral regurgitation, and in six explanted hearts as the result of dilated cardiomyopathy. mRNA levels were determined by solution hybridization.

β 1 ligand activity was decreased in the cases of mitral regurgitation ($p < 0.01$) and dilated cardiomyopathy ($p < 0.001$). β 2 ligand activity did not differ between the three groups. mRNA expression was depressed in mitral regurgitation regarding both β 1 ($p < 0.001$) and β 2 ($p < 0.01$), while no differences were observed in dilated cardiomyopathy as compared to the donor hearts.

The regulation of β 1 and β 2 adrenoceptor ligand activity and mRNA expression appears to follow a specific pattern in dilated cardiomyopathy. The specific down-regulation of β 1 ligand activity seems to occur at a posttranslational level.

Key words chronic heart failure; myocardium; messenger RNA; ligand; adrenoceptor; subunit

Severe heart failure is associated with a chronically increased sympathetic tone. A common feature is that the more advanced the disease is, the more blunted the inotropic response of the myocardium, and the density of betareceptors is also decreased.¹⁾ There are conflicting reports as to whether in severe chronic heart failure β 1 and β 2 adrenoceptor (BAR-1 and BAR-2) expressions are equally attenuated or whether mainly the BAR-1 expression is down-regulated. In end-stage dilated cardiomyopathy, a selective loss of BAR-1 has been reported while the BAR-2 level has been reported to be unaltered.²⁾ In end-stage ischemic cardiomyopathy, Bristow and co-workers³⁾ reported a selective down-regulation of BAR-1, although the decrease was less pronounced than in dilated cardiomyopathy. On the other hand, Brodde and co-workers⁴⁾ and Steinfath and co-workers⁵⁾ reported that ischemic cardiomyopathy was characterized by a similar degree of down-regulation of the two receptor subtypes. In mitral valve disease, a number of authors have reported that with progressive heart failure, both receptor subtypes are down-regulated.¹⁾

By using a solution hybridization assay, the mRNA of BAR-1 and BAR-2 can be quantitated from small myocardial tissue samples.⁶⁾ In donor hearts, similar levels were reported for the mRNA of BAR-1 and BAR-2 in atrial, right and left ventricular biopsies. BAR mRNA levels in papillary muscles from patients with mitral stenosis did not differ from donor hearts. By contrast, but in agreement with ligand binding studies showing decreased BAR-1 and BAR-2 receptor densities, volume loaded papillary muscles from patients with mitral regurgitation showed decreased levels of mRNA in both BAR-1 and BAR-2.

Against this background, with a specific down-regulation of BAR-1 ligand activity in dilated cardiomyopathy, it was hypothesised that BAR-1 mRNA levels

should be decreased. Hearts explanted due to end-stage dilated cardiomyopathy and papillary muscles obtained at surgery from patients with mitral regurgitation were studied. The ligand activity and mRNA levels of BAR-1 and BAR-2 in the left ventricle were compared to donor hearts without any known heart disease.

MATERIALS AND METHODS

Donor Heart Myocardial samples were obtained from six brain dead kidney and liver donors (18–40 years old). The heart weight range was 210–390 g. According to patients' records, none of the organ donors had any known heart disease or had used any drugs. Myocardial samples were obtained within 20 h of the diagnosis of brain death, and samples were taken from the heart within 15–30 min after the heart had stopped beating.

Mitral Regurgitation Papillary muscles were obtained from seven patients subjected to mitral valvectomy due to mitral regurgitation. On a 4-point scale, mitral regurgitation was quantified as points 3 and 4 in five patients (52–65 years old) and point 2 in two patients in whom mitral regurgitation was complicated by appreciable mitral stenosis. The New York Heart Association (NYHA) functional classifications were I ($n = 1$), II ($n = 3$) and III ($n = 3$). One patient had a history of hypertension and two of previous acute myocardial infarction. Pharmacological treatment in these patients included digoxin, diuretics, bronchodilators, verapamil, and angiotensin converting enzyme inhibitors. No patients were on beta-adrenergic blockers.

Dilated Cardiomyopathy Cardiomyopathic hearts were obtained from six patients (26–56 years old) with biventricular end-stage heart failure, who were undergoing orthotopic heart transplantation. The NYHA functional classification ranged from IIIb to IV, and the cardiac index

* To whom correspondence should be addressed.

from 1.0–3.5 l/min/m². The ejection fraction was 0.23 ± 0.05 , significantly ($p < 0.0001$) less than that found in the patients operated on for mitral valve disease. Pretransplant testing which included endomyocardial biopsy was used to exclude specific causes of cardiomyopathy, especially hemochromatosis, thyrotoxicosis and connective tissue disease. Medical therapy consisted of digoxin, diuretics, and nitrates. None of the patients received beta-adrenergic antagonists or agonists.

Preparation of Tissue and Nucleotides From both the donor hearts and cardiomyopathic hearts, the part analyzed was the subendocardial muscle at the base of the left ventricular papillary muscle. Each sample was immediately frozen in liquid nitrogen and stored at -70°C until analysis. Frozen biopsies were dissected free of visible blood, connective tissue and extracellular fat. Total nucleic acids were prepared by digestion of the homogenized myocardium with proteinase K ($100 \mu\text{g} \cdot \text{ml}^{-1}$) in a buffer containing 1% sodium dodecyl sulphate (SDS), 10 mmol·l Tris (pH 7.5), and 5 mmol·l EDTA with subsequent extraction with phenol–chloroform.⁶⁾

Preparation of Hybridization Probes Complementary oligonucleotide cRNA probes (50 bases) were prepared and characterized as previously described.⁶⁾ Briefly, oligonucleotides were synthesized and cloned into Pst I/Hind III sites in pGEM-3 (Promega Biotechnology, Madison, WI, U.S.A.). The sequence of the insertion was confirmed by DNA sequencing using the dideoxy chain termination method. Plasmid was employed for the *in vitro* synthesis of cRNA using SP6 RNA polymerase, and for the opposite mRNA strand using T7 RNA polymerase. The probes were radiolabelled with [³⁵S]-uridine triphosphate [³⁵S]UTP. The sequence of the oligonucleotides used was: for the β_1 adrenoceptor nucleotides 739–789: 5'GTGTTCCGCGAGGCCAGAGGAGGTGAAGAAGATCGACAGCTGCGAGCGC3';²⁾ and for the β_2 adrenoceptor nucleotides 772–822: 5'CTCGCAGATCTTCCAAGTTCTGCTTGAAGGAGCACAAAGCCCTCAAGACG3'. In addition, the opposite mRNA strands of these probes were synthesized *in vitro* and used as standards. These cRNA probes have been extensively characterized in our laboratory, including specificity, as tested in Northern blot hybridization analysis.⁷⁾ Figure 1 shows one distinct band in the specimen from human liver and adipose tissue. Testing was done in these tissues due to the scarcity of human myocardial materials.

Northern Blot Hybridization This assay was performed essentially as described by Maniatis and co-workers.⁷⁾ RNA samples (20–30 μg) were denatured for 10 min at 65°C in dimethylsulphoxide (50%), formaldehyde (2.2 mol/l), sodium phosphate (10 mmol/l, pH 7.5) and EDTA (0.5 mmol/l), and then electrophoresed through an agarose gel (1.2%) containing formaldehyde (2.2 mol/l) in sodium phosphate (10 mmol/l; pH 7.5) for 500 V·h in a running buffer containing morpholinopropane sulphonic acid (0.04 mmol/l, pH 7.4), sodium acetate (10 mmol/l), and EDTA (1 mmol/l). The integrity of RNA samples was verified after gel electrophoresis by ethidium bromide staining. After two 30-min equilibrations in $20\times\text{SSC}$ (3 mol/l of NaCl, 0.3 mol/l of sodium citrate), the RNA

Fig. 1. Northern Blot Analysis of RNA from Human Liver (L) and Adipose (A) Tissue

Two samples (each containing 20 μg of total RNA) were electrophoresed on the same gel and blotted to the same nylon filter which was cut into two parts. Each half was subjected to RNA blot analysis and probed with ³²P-labeled BAR-1 and BAR-2 cRNA probes as described in Materials and Methods. Positions of RNA markers are indicated. One experiment out of two is depicted.

was transferred by the Southern technique to hybond-N filters (Amersham Corp., Buckinghamshire, UK). Nylon membranes to which RNA had been transferred were then prehybridized for 2 h at 55°C in 50% formamide, $5\times\text{SSC}$ (1.0 mol/l of NaCl, 0.1 mol/l of sodium citrate), $5\times\text{Denhardt's}$ solution (0.02% of Ficoll, 0.02% of polyvinyl pyrrolidone, and 0.02% of bovine serum albumin), 5 mmol/l of phosphate buffer (pH 6.5), 5 mmol/l of SDS and 200 $\mu\text{g}/\text{ml}$ of salmon testis DNA. Hybridization was carried out for 40 h at 55°C in an identical solution containing $4\text{--}6 \times 10^2$ cpm/ml of ³²P-labeled cRNA probe. After hybridization, the filter was washed serially with $0.1\times\text{SSC}$ and 0.1% of SDS at 68°C to eliminate non-specific binding of the probes to the filter and ribosomal RNA. Autoradiographs were obtained by exposure to Kodak XAR-5 film with an intensifying screen for 48 h at 70°C . Molecular weight standards corresponding to RNA species of 28S and 18S were prepared from MCF7 cells. RNA for BAR-1 and BAR-2 were electrophoresed on the same gel and blotted over to the same filter. The filter was cut into different lanes for subsequent hybridization to either the BAR-1 probe or the BAR-2 probe.

Solution hybridization was carried out as described previously.⁶⁾ Briefly, RNA hybrids were allowed to form at 70°C in a buffer consisting of $0.6 \text{ mol} \cdot \text{l}^{-1}$ NaCl, $30 \text{ mmol} \cdot \text{l}^{-1}$ EDTA, 0.1% SDS, $10 \text{ mmol} \cdot \text{l}^{-1}$ dithiothreitol, and 25% formamide. After overnight incubation, samples were treated with ribonuclease (RNase) for 45 min. RNase resistant radioactivity was precipitated by trichloroacetic acid and collected on glass fibre filters. Sample hybridization was compared to the *in vitro*

synthesised mRNA strand (complementary to the radioactive probe). Each sample was analyzed in duplicate, and the amount of mRNA was related to the total amount of total nucleic acids (TNA) in the sample as determined spectrophotometrically. The specificity of the method has previously been characterized with no cross-reactivity between the probes and with the linearity of the assays.⁸⁾

Beta-adrenergic Binding Characteristics Binding assays were carried out on left ventricular biopsies in a buffer (pH 7.4): 25 mM Tris–75 mM MgCl₂. Saturation-binding isotherms were obtained by incubating the membranes for 2 h with varying concentrations of ICYP (12.5–400 pM) at 30 °C. Specific binding was defined as that displaced by 1 μM CGP 12177 (beta-adrenergic binding). Determinations of BAR-1 and BAR-2 were made according to the inhibition of ICYP binding by the selective BAR-1 antagonist CGP 20712A. The reaction was terminated by dilution with 5 ml of an ice-cold buffer. The samples were then poured over GF/C 2.5 cm glass microfiber filters (Whatman International, Ltd., England) under reduced pressure, followed by washing with 15 ml of buffer. Filters were counted in a Compugamma universal counter (LKB, Sweden). The binding parameters, $B_{\max}$ and K_d , were determined by a computer-assisted nonlinear least squares fitting program as previously described. All data were handled with Macintosh hardware using the software Excel 3.0 (Microsoft, Inc.) and KaleidaGraph 3.0 (Abelbeck Software, Inc.).

Statistics and Ethics The values presented are means with a standard error of the mean. Statistical non-homogeneity between the groups was tested by analysis of variance. Differences between groups were localized by Fisher's protected least significance difference test. The study was approved by the ethics committees of the Karolinska Institute and of the Semelweiss Institute and by the Hungarian Ministry of Health.

RESULTS

Expressions of BAR-1 and BAR-2 receptor densities were measured in radioligand experiments using the non-selective BAR antagonist CGP 12177 and the BAR-1 selective antagonist CGP 20712A. In confirmation of previous reports,^{1–5)} Fig. 2 shows that BAR-1 density in dilated cardiomyopathy was depressed as compared to donor hearts (7.4 ± 2.2 vs. 34.3 ± 3.8 fmol/mg protein, $p < 0.001$), while no difference was observed for BAR-2 (26.2 ± 7.6 vs. 25.8 ± 5.2 fmol/mg protein). In mitral regurgitation, BAR-1 was also depressed (15.2 ± 3.8 vs. 34.3 ± 3.8 fmol/mg protein, $p < 0.01$), while BAR-2 showed an insignificant tendency toward decreased activity (15.9 ± 6.7 vs. 25.8 ± 5.2 fmol/mg protein, $p < 0.25$).

Steady state levels of mRNA were determined by solution hybridization on left ventricular subendocardial samples taken at the base of the papillary muscle. As shown in Fig. 3 specimens from dilated cardiomyopathy did not show any difference compared to donor hearts regarding both BAR-1 (30.3 ± 2.9 vs. 29.2 ± 4.1 amol/μg TNA, $p = \text{NS}$) and BAR-2 mRNA (26.9 ± 3.1 vs. 23.4 ± 3.3 amol/μg TNA, $p = \text{NS}$). By contrast, in mitral regurgitation both BAR-1 mRNA (3.2 ± 0.4 vs. 29.2 ± 4.1

Fig. 2. Left Ventricular BAR-1 (Above) and BAR-2 (Below) Receptor Densities (fmol/μg protein) in Left Ventricular Samples of Donor Hearts ($n=6$), Papillary Muscles from Patients Operated for Mitral Regurgitation (MR, $n=7$), and Explanted Hearts Due to End-Stage Dilated Cardiomyopathy (DCM, $n=6$)

Values are given as mean with S.E.M. **, $p < 0.01$ and ***, $p < 0.001$ relative to donor hearts. NS denotes non-significant.

Fig. 3. Left Ventricular mRNA (amol/μg TNA) of BAR-1 (Above) and BAR-2 (Below) of Donor Hearts ($n=6$), Papillary Muscles from Patients Operated on for Mitral Regurgitation (MR, $n=7$) and Explanted Hearts from Patients with End-Stage Dilated Cardiomyopathy (DCM, $n=6$)

Values are given as mean with S.E.M. **, $p < 0.01$ and ***, $p < 0.001$, relative to donor hearts.

amol/μg TNA, $p < 0.001$) and BAR-2 mRNA (11.5 ± 3.3 vs. 23.4 ± 3.3 amol/μg TNA, $p < 0.01$) species were depressed compared to the donor hearts.

DISCUSSION

Unexpectedly, the left ventricular BAR-1 mRNA steady

state level was unaltered in dilated cardiomyopathy in the presence of a decreased BAR-1 ligand-determined density, the latter taken as an index of functional protein content. As previously reported⁶⁾ and also observed in the present investigation, both BAR-1 and BAR-2 mRNA levels were decreased in mitral regurgitation. These lower mRNA levels appear to occur in parallel with a decrease in receptor densities. In the present investigation, BAR-1 density was decreased and BAR-2 density tended to decrease, but the results were non-significant, probably due to a statistical β -error due to the small group investigated. These results are in keeping with previous reports suggesting that both receptor subtypes are down-regulated in mitral regurgitation.¹⁾

It could be argued that donor hearts do not represent the normal heart. Even though no signs of cardiac disease were present, the myocardium of donor hearts might not represent the normal human myocardium due to the stress imposed by the trauma the patient has suffered and due to the intensive care that has been given until explantation. These arguments may be valid, although it must also be stated that these human hearts are the best reference material available. The relevance of the use of donor hearts as reference material when studying dilated cardiomyopathy is also illustrated by the fact that the myocardium from patients with mitral regurgitation had lower levels of both BAR receptor densities and steady state mRNA than the donor hearts.

The mismatch between receptor density and BAR-1 mRNA level in dilated cardiomyopathy is an intriguing observation and may relate to receptor reserve, turnover and degradation of the receptor protein. In human myocardium, the receptor reserve for BAR is restricted as compared to the rat.⁹⁾ In glioma cells it has been shown that following isoprenaline stimulation, BAR-1 receptor proteins had a higher turnover than BAR-2 receptor proteins, while mRNA levels were unaltered.¹⁰⁾ Such factors may explain the down-regulation of BAR-1 and BAR-2 in the chronically stressed myocardium, but are not sufficient to explain the differences between dilated cardiomyopathy and mitral regurgitation. Compared to mitral regurgitation and in spite of a worse NYHA functional class and left ventricular function in patients with dilated cardiomyopathy, only the BAR-1 ligand determined receptor density, and the mRNA were depressed in dilated cardiomyopathy. Another factor relates to the role of immunogenic factors. Dilated cardiomyopathy has been linked to specific HLA-DR phenotypes (HLA-DR4/DR1)¹¹⁾ and the presence of specific antibodies.¹¹⁾ Of patients with dilated cardiomyopathy, 30–40% have circulating antibodies directed specifically against BAR-1. These antibodies not only bind to BAR-1, but they also stimulate and cause an enhanced turnover of the receptor protein. Experimentally, desensitization following exposure to antibodies is almost never associated with a recovery or recycling of receptors as is the case following isoproterenol-induced desensitisation.¹²⁾ Maintained or even increased BAR-1 mRNA levels may therefore be reactions to an increased protein turnover.

These observations demonstrate for the first time at the

mRNA level a specific and principally different tissue response regarding β -adrenoceptor expression in dilated cardiomyopathy as compared to mitral regurgitation. While we used solution hybridization for the determination of mRNA, and found in dilated cardiomyopathy similar levels to those in donor hearts, other investigators using a polymerase chain reaction reported increased¹³⁾ or decreased^{2,14)} levels as compared to the controls. Such differences could be due to differences in methodology or reference materials. Our biopsies were dissected free from visible blood, extracellular fat and connective tissue. Bristow and co-workers²⁾ used myocardial specimens from the middle of the free ventricular wall and Ungerer and co-workers¹²⁾ used left ventricular tissue with the exclusion of the papillary muscles. Bristow and co-workers²⁾ have also argued that our method may be non-specific and therefore it gives falsely high results. They used, in parallel to quantitative PCR, an RNase protection assay with cDNA fragments larger than 200 bp. We used a solution hybridization based on cRNA with a fragment size of 50 bp. Our probe was specific in Northern blot hybridization analysis and the absence of cross hybridization between BAR-1 and BAR-2 mRNA has previously been shown with solution hybridization.⁸⁾ The differential response between samples from dilated cardiomyopathy and from mitral regurgitation also indicates the specificity of our method.

Our results suggest that in papillary muscles from patients operated on for mitral regurgitation BAR-1 and BAR-2 are down-regulated before translation, while in dilated cardiomyopathy a posttranslational reduction in BAR-1 protein activity and steady BAR-2 expressions are manifest. These results add to the previously reported specific phenotype in dilated cardiomyopathy. The observed adaptations in this study could be only one aspect in a complex disease process in dilated cardiomyopathy. Not only BAR function but also the guanine nucleotide binding proteins (G-proteins) are affected. These are important integral parts of the adrenergic transduction pathway. An increased activity of the G_i but not the G_s protein in dilated cardiomyopathy has been demonstrated to be of functional importance for the uncoupling of contractility.¹⁵⁾ Although both G_s -alpha and G_i -alpha 3 mRNA levels are increased, G_s and G_i protein contents are not increased in dilated cardiomyopathy.¹⁶⁾ The increased functional activity of G_i thus appear to be regulated at the posttranslational or protein-protein interaction level. Upregulation of adenine nucleotide translocator mRNA and down-regulation of creatine kinase B subunit protein¹⁷⁾ further add to the view that a differentiated network of regulatory factors are involved in dilated cardiomyopathy phenotype expression.

Acknowledgements This study was supported by the Swedish Medical Research Council 4494, 9515, and 9522 and the Swedish Heart and Lung Foundation, The Swedish Medical Association.

REFERENCES

- 1) Brodde O. E., *Pharmacol. Rev.*, **43**, 203 (1990).
- 2) Bristow M. R., Minobe W. A., Raynolds M. V., Port J. D., Rasmussen R., Ray P. E., Feldman A. M., *J. Clin. Invest.*, **92**, 2737 (1993).
- 3) Brodde O. E., Zerkowski J. F., Borst H. G., Maier W., Michael M. C., *Eur. Heart J.*, **10**, Suppl. B, 38 (1989).
- 4) Steinfath M., Lavicky J., Schmitz W., Scholz H., Doring V., Kalmar P., *Eur. J. Clin. Pharmacol.*, **42**, 607 (1992).
- 5) Bristow M. R., Anderson F. L., Port J. D., Skerl L., Hershberger R. E., Larrabee P., O'Connell J. B., Renlund D. G., Volkman K., Murray J., Feldman A. M., *Circulation*, **84**, 1024 (1991).
- 6) Sylvén C., Arner P., Hellström L., Jansson E., Sotonyi P., Somogyi A., Brönnegård M., *Cardiovasc. Res.*, **25**, 737 (1991).
- 7) Maniatis T., Fritsch E. F., Sambrook J., "Molecular Cloning—A Laboratory Manual," Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, 1982.
- 8) Arner P., Hellström L., Wahrenberg H., Brönnegård M., *J. Clin. Invest.*, **86**, 1595 (1990).
- 9) Bals S., Sohlmann W., Zerkowski H. R., Michel M. C., Brodde O. E., *J. Cardiovasc. Pharmacol.*, **19**, 222 (1992).
- 10) Fishman P. H., Miller T., Curran P. K., Feussner G. K., *J. Recept. Res.*, **14**, 281 (1994).
- 11) Limas C. J., Goldenberg I. F., Limas C., *Am. Heart J.*, **122**, 108 (1991).
- 12) Magnusson Y., Marullo S., Hoyer S., Waagstein F., Andersson B., Vahlne A., Cuillet J. G., Strosberg A. D., Hjalmarson Å., Hoebeke J., *J. Clin. Invest.*, **86**, 1658 (1990).
- 13) Ihl-Wahl R., Marquetant R., Strasser R. H., *Circulation*, **86**, I-767 (1992).
- 14) Ungerer M., Böhm M., Elce J. S., Erdmann E., Lohse M. J., *Circulation*, **87**, 454 (1993).
- 15) Böhm M., Gierschik P., Jakobs K-H., Pieske B., Schnabel P., Ungerer M., Erdman E., *Circulation*, **82**, 1249 (1990).
- 16) Fu L. X., Waagstein F., Hoebeke J., Liang Q. M., Sylvén C., Jansson E., Sotonyi P., Hjalmarson Å., *Cardiovasc. Res.*, **26**, 950 (1992).
- 17) Sylvén C., Jansson E., Sotonyi P., Somogyi A., Fu L-X., Waagstein F., Hjalmarsson Å., Brönnegård M., *Cardiovasc. Res.*, **27**, 1295 (1993).