Marked enhancement in myocardial function resulting from overexpression of a human β -adrenergic receptor gene

Transgenic mice with intense cardiac expression of a human β -adrenergic receptor gene were engineered and shown to display marked improvements in baseline myocardial and left ventricular function. Heart/body weight ratios and histologic appearance were not found to be significantly altered, suggesting that receptor gene expression did not induce pathologic changes. Given the substantial reduction in β -adrenergic receptor density and resultant reduction in inotropic responsiveness observed in chronic heart failure, these findings represent a novel approach for increasing myocardial function with important clinical implications. (J THORAC CARDIOVASC SURG 1995;109:236-41)

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The primary physiologic mechanism for enhancing myocardial performance during stress involves stimulation of myocardial β -adrenergic receptors (β -ARs) by endogenous catecholamines. Furthermore, the main therapeutic approach for augmenting myocardial function involves administration of synthetic agonists, which stimulate myocardial β -ARs (e.g., dopamine and dobutamine). However, in a number of cardiac disease states that result in chronic congestive heart failure, myocardial β -AR function is impaired and β_1 -AR density markedly reduced.¹ This impairment in the myocardial β -AR system significantly reduces inotropic responsiveness and may further limit the ability of the myocardium to compensate for the primary disease process.¹

We² have previously described the ability to target expression of an exogenous β -AR gene to the myocardium of transgenic mice. Coupling the deoxyribonucleic acid (DNA) coding sequence of the human β_2 -AR gene to an α -myosin heavy chain promoter sequence can achieve intense myocardium-specific expression of the exogenous β -AR.^{2,3} In this report, enhancement of myocardial function induced by this β -AR gene expression is described in detail. In addition, morphologic and histologic characterization of the transgenic hearts is presented. Finally in the context of the known reduction in β -AR number in chronic heart failure,¹ potential therapeutic implications of this work are discussed.

Methods

DNA construct and generation of transgenic animals. The DNA construct used for generation of the transgenic mice consisted of the human β_2 -AR coding sequence coupled to the α -myosin heavy chain promoter.^{2,3} This DNA was microinjected into the pronuclei of fertilized single cell mouse embryos, and the embryos were reimplanted into the oviduct of an adult female mouse.⁴ Offspring were screened for the presence of the exogenous gene by Southern analysis of DNA extracted from tail cuts, and founder mice were identified. These founders were bred with normal mice to generate a line of transgenic mice; two lines of transgenic mice that contain the exogenous gene (TG4 and TG33) are described in this report. All animal manipulations were conducted after appropriate anesthesia in accordance with institutional review board and National Institutes of Health guidelines (NIH Publication No. 86-23, revised 1985). All data presented were obtained on either transgenic or control adult mice, approximately 2 months of age.

Ligand binding assay. Excised hearts were placed in ice cold lysis buffer (Tris HCl, 5 mmol/L [pH 7.4], and EDTA,* 5 mmol/L) and homogenized. After an initial 500 g centrifugation, the supernatant was removed and centrifuged at 40,000 g and the resultant pelleted membrane fraction was resuspended in binding buffer (Tris HCl, 75 mmol/L [pH 7.4], MgCl₂, 12.5 mmol/L, and EDTA, 2 mmol/L.) Saturation binding assays were then performed on the membrane fractions in the presence of [¹²⁵I]-cyanopindolol, 500 pmol/L (a β -AR-specific ligand); non-specific binding was determined in the presence of alprenolol, 20 μ mol/L. Reactions were conducted at 37° C for 1 hour and then terminated by vacuum suction through glass fiber filters. Filters were counted in a gamma counter and receptor density was expressed as mean membrane protein (picomoles per milligram) plus or minus standard error of the mean.⁵

Immunohistochemical labeling. Frozen sections were cut at 7 μ m for indirect immunofluorescence studies. Sections were rinsed three times for 3 minutes in phosphate-buffered saline solution (PBS) and 3 minutes in

PBS with 0.05% Triton X-100 (Triton-PBS), blocked with serum diluent (10% goat serum in PBS with 0.1% bovine serum albumin and 0.1% sodium azide), and then rinsed for 15 minutes in Triton-PBS before overnight incubation at 4° C with a primary rabbit antihuman β_2 -AR antiserum⁶ (1:500 dilution in serum diluent). The sections were then washed four times for 10 minutes in Triton-PBS at room temperature and incubated for 1 hour in fluorescein isothiocyanate-conjugated goat antirabbit immunoglobulin G (1:50 dilution in serum diluent). After five 3-minute rinses in PBS, the sections were mounted with sodium iodide (25 gm/L) in 1:1 PBS/glycerol solution and photographed.

Isolated myocardial function. After excision of the heart, the atria were dissected and suspended at optimal resting tension in an organ bath in carbogenated modified Krebs bicarbonate solution (NaHCO₃, 25 mmol/L [pH 7.4]; NaCl, 118 mmol/L; KCl, 4.8 mmol/L; MgSO₄·7H₂O, 1.2 mmol/L; KH₂PO₄, 1.2 mmol/L; CaCl₂·2H₂O, 1.75 mmol/L; glucose, 10 mmol/L; Na₂S₂O₅, 0.1 mmol/L; EDTA, 0.03 mmol/L), supplemented with ascorbic acid (1.1×10^{-4} mol/L), cocaine (1×10^{-5} mol/L), corticosterone (4×10^{-5} mol/L), and phentolamine (3×10^{-6} mol/L). Left atria were paced with a pulse duration of 3 msec and the voltage set at threshold plus 20%; rates were not different between the two groups. Isometric developed tension was measured at baseline and after administration of isoproterenol, 3×10^{-8} mol/L, the dose that effected a maximum response. Data are given as the means plus or minus standard error of the mean.

In vivo cardiac function. After anesthesia, a cervical incision was performed, the trachea intubated, and the animal connected to a volume-cycled ventilator. A carotid artery was then cannulated with a flame-stretched PE 50 catheter connected to a modified P50 Statham transducer (Viggo Spectramed Inc., Critical Care Div., Oxnard, Calif.). The chest and pericardium were then opened and a 2F high-fidelity micromanometer catheter (Millar Instruments, Inc., Houston, Tex.) was inserted into the left atrium, advanced across the mitral valve, and secured in the left ventricle (LV). Continuous aortic pressures, LV systolic and diastolic pressures, and the derivative of LV pressure (LV dP/dt) were recorded on an eight-channel chart recorder and in digitized form on computer disk for beat averaging (codas, Dataq Instruments, Akron, Ohio).⁷ Ten sequential beats were averaged for each measurement (CORDAT, Essen, Germany) and the data were expressed as the mean plus or minus standard deviation. In separate experiments, these parameters were recorded both at baseline and 4 minutes after the systemic administration of 5 μ g of ICI-118551, a β_2 -AR antagonist.

Heart/body weight ratios and histologic evaluation. Wet heart/body weight ratios were determined as previously described and expressed as the mean plus or minus standard deviation.⁸ Hearts were then fixed in a 1% formaldehyde solution and processed for paraffin embedding. LV sagittal sections were then generated, labeled with wheat-germ agglutinin, and myocyte cross-sectional areas were determined.⁸

Atrial natriuretic factor (ANF) Northern blots. Ventricular ribonucleic acid (RNA) was extracted, fraction-

*Ethylenediaminetetraacetic acid.

Fig. 1. Immunohistochemical labeling of the myocardium was performed on longitudinal ventricular sections with antisera specific for the human β_2 -AR: **A**, Control nontransgenic mouse. **B**, TG4 transgenic mouse. *Inset* displays the mean β -AR densities for four control versus four TG4 hearts.

Fig. 2. Isometric developed tension was measured for control ($n = 8$), TG4 transgenic ($n = 4$), and TG33 transgenic ($n = 4$) isolated left atria. All atria were paced at similar rates. The *asterisks* indicate $p < 0.05$, Student's t test, for the transgenic baseline or isoproterenol stimulated tension value relative to the respective control value.

ated on a 1% agarose formaldehyde gel, and transferred to a nitrocellulose membrane as previously described.⁸ Nitrocellulose membranes were prehybridized and then hybridized with a random-primer radiolabeled ANF cDNA probe⁸; blots were stripped and reprobbed with the rat glyceraldehyde 3-phosphate dehydrogenase cDNA probe (Ambion, Austin, Tex.).

Results and discussion

Immunohistochemistry performed on ventricular myocardial sections with an antiserum specific for the human β_2 -AR demonstrated impressive labeling of the transgenic myocardium with virtually no labeling of control tissue (Fig. 1). β -AR density was quantitated by radioligand binding and was found to be increased approximately 175-fold in the TG4 animals over control levels (Fig. 1, *inset*). This level of receptor overexpression results from the α -myosin heavy chain promoter, which is a strong myocardial promoter.^{2,3}

Measurement of isometric tension development is a specific test of myocardial function and eliminates potentially confounding differences resulting from alterations in preload or afterload. Isolated mouse atria provide a stable preparation for the quantitation of isometric tension development. Atria from two separate lines of transgenic mice (TG4 and TG33) were compared to control atria (Fig. 2), and markedly enhanced baseline tension (greater than threefold increase) was present in the transgenic animals relative to control animals. Isometric tension after isoproterenol administration was also significantly greater in the transgenic myocardium relative to control values. The transgenic myocardial response to isoproterenol was minimal, suggesting that biochemical elements of contractility beyond the β -AR system become limiting such as calcium channels or myosin adenosine triphosphatase activity.

Fig. 3. **A**, Baseline LV dP/dt_{max} was determined for control mice ($n = 7$) and TG4 transgenic mice ($n = 7$). The asterisks indicate $p < 0.05$, Student's t test, relative to control. **B**, In separate experiments, LV dP/dt_{max} was determined in TG4 transgenic mice ($n = 5$) at baseline and 4 minutes after the systemic administration of 5 μ g of ICI-118561 (a β -AR antagonist). Asterisks indicate $p < 0.05$, paired Student's t test.

The development of microsurgical techniques has allowed for in vivo measurements of LV hemodynamics,⁷ and baseline parameters were measured in TG4 transgenic mice and compared with control mice. Heart rates were significantly elevated in the transgenic mice relative to control mice (441 ± 44 versus 349 ± 21 beats/min, respectively; $n = 7$); mean aortic pressures and LV end-diastolic pressure, however, were not different. Under these conditions, LV maximal dP/dt (LV dP/dt_{max}) is an accurate parameter of LV performance, and in the transgenic animals, significant increases in LV dP/dt_{max} were present relative to control values (Fig. 3, A).

To demonstrate that the enhanced LV function resulting from the β -AR overexpression could be regulated in vivo, we measured LV dP/dt_{max} before and after the systemic administration of the β_2 -AR antagonist, ICI-118551. Marked decreases in the elevated baseline LV dP/dt_{max} were effected in all transgenic animals (Fig. 3, B); control animals demonstrated no significant change in response to the administration of this agent (data not shown). This study demonstrates that the functional enhancement in these transgenic animals can be readily

Table I

	Control	TG4 (β_2 -AR)
Heart/body weight ratio (mg/gm)	5.65 ± 0.55 ($n = 11$)	5.94 ± 0.63 ($n = 11$)
Ventricular myocyte cross-sectional area (μm^2)	208 ± 43 ($n = 5$)	233 ± 30 ($n = 5$)

Heart/body weight ratios and LV myocyte cross-sectional areas were compared between TG4 transgenic and control mice. For myocyte cross-sectional areas, five hearts were studied for each group and for each heart a mean cross-sectional area was determined from the measurement of 100 LV free wall myocytes; the average of the 5 means $\pm$ the standard deviation is shown for each group. For both parameters, no significant difference was detected between the transgenic and the control values ($p > 0.05$, Student's t test).

modulated by the systemic administration of a specific β -AR antagonist; the molecular mechanisms by which this antagonist effects these changes probably involves more than the displacement of endogenous catecholamines from the overexpressed receptors.^{9*}

Given the chronic, marked functional increases that were present in these transgenic mice, animals

*Bond RA, Johnson TD, Milano CA, et al. Demonstration of inverse agonists in transgenic mice overexpressing the β_2 -adrenoceptor. Unpublished data.

Fig. 4. ANF Northern blot analysis. Each lane was loaded with 8 μ g of ventricular total RNA and represents a different animal: control nontransgenic ($n = 2$), TG4 transgenic ($n = 5$), and mice with genetically induced ventricular hypertrophy (positive control, $n = 2$). The blot was probed first with an ANF cDNA probe and subsequently with a GAPDH cDNA probe to demonstrate equivalent RNA loading.

were evaluated for the development of cardiac disease. Heart/body weight ratios were minimally altered when a large group of transgenic animals were compared with controls (Table I). LV myocyte cross-sectional areas were not significantly increased in the transgenic hearts relative to controls, confirming the absence of hypertrophy (Table I). Furthermore, fibrosis or myocyte necrosis was not present in transgenic myocardial sections. Finally, ventricular ANF mRNA levels, a sensitive molecular marker associated with pressure-overload hypertrophy,¹⁰ were assessed by Northern blot analysis (Fig. 4). A very weak ANF signal was present on Northern blots of normal control ventricular mRNA and mRNA from five TG4 animals. Conversely, mRNA from animals with genetically induced ventricular hypertrophy⁸ characteristically had a very strong signal. Equivalent signals obtained with the glyceraldehyde 3-phosphate dehydrogenase probe excluded unequal mRNA loading or degradation. Together, these data suggest that the functional enhancement generated by β -AR overexpression does not effect significant morphologic or histologic alteration. Furthermore, the normal myocyte gene expression appears unaltered.

In this report, overexpression of an exogenous β -AR gene in the myocardium is presented as a

novel means of enhancing myocardial and ventricular function. Deleterious histologic or morphologic changes did not appear to be associated with this augmented function. Furthermore, these functional increases could be regulated by the systemic administration of a specific β_2 -AR antagonist.* More generally, this report highlights the utility of transgenic animals generated with myocardium-specific promoters for studying the effects of altered gene expression on myocardial function.

Recent studies have demonstrated the ability of recombinant, replication-deficient adenovirus to transfer genes to the myocardium of adult experimental animals and effect significant levels of expression.¹¹ Such an approach may be useful for myocardial β -AR gene transfer and may achieve increased levels of receptor expression capable of enhancing cardiac function. Furthermore, given the specific defects in the β -AR system in patients with chronic congestive heart failure, therapeutic efforts to transfer β -AR genes to the myocardium in vivo represent a promising area for future clinical intervention.

*Bond RA, Johnson TD, Milano CA, et al. Demonstration of inverse agonists in transgenic mice overexpressing the β_2 -adrenoceptor. Unpublished data.

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Discussion

Dr. Stanley K. C. Tam (*Boston, Mass.*). In heart failure β -adrenergic receptors are down-regulated, presumably to protect the myocardium from elevated levels of adrenergic stimuli. What do you think overexpression of this same receptor in a failing heart will do?

Is α -myosin heavy chain up-regulated or down-regulated in heart failure as opposed to β -myosin heavy chain?

Dr. Milano. Reports have not demonstrated that down-regulation of β -adrenergic receptors represents a protective effect, and we believe that this down-regulation may inhibit the ability of the myocardium to compensate for the primary disease that is causing the impairment of myocardial function.

The α -myosin heavy chain may be down-regulated in heart failure, but this is a species-dependent process. However, other promoter sequences could be used to overexpress the β -adrenergic receptor.

Mr. Thoralf M. Sundt (*Harefield, England*). Two bands appeared to be expressed on your Northern blot. Is that correct, or was I misreading your blot?

Dr. Milano. There is one predominant band of the appropriate size, which is roughly 2.4 kb. I think the lower labeling represents some degradation of the RNA on that blot.

Dr. Andrew S. Wechsler (*Richmond, Va.*). I was surprised that in the isolated atria there appeared to be enhanced intrinsic contractile function, which almost looked greater than the increment that you got with a β -adrenergic agonist. Were you surprised by that or can you explain that?

Dr. Milano. Initially we thought we would find a greater response to isoproterenol rather than an increase in baseline function. However, what seems to be happening is that when the receptor is so greatly overexpressed, the percentage of the receptor that remains in the activated state even in the absence of agonist (for example, isoproterenol or catecholamine in the sense of the in vivo setting) becomes significant. Since there is such a large pool of receptors in these transgenic animals, the small fraction that remains activated becomes significant and results in a completely activated system in the absence of agonists.