Proc. Natl. Acad. Sci. USA
Vol. 93, pp. 10483–10488, September 1996
Pharmacology

Myocardial signaling defects and impaired cardiac function of a human β_2-adrenergic receptor polymorphism expressed in transgenic mice

(adenylyl cyclase/mutation/G protein/catecholamine/heart failure)

JAMAL TURKI*, JOHN N. LORENZ†, STUART A. GREEN*, ELIZABETH T. DONNELLY*, MARIE JACINTO*, AND STEPHEN B. LIGGETT*‡§

Departments of *Medicine, ‡Molecular Genetics, and †Physiology, University of Cincinnati College of Medicine, Cincinnati, OH 45267

Communicated by Robert J. Lefkowitz, Duke University Medical Center, Durham, NC, July 2, 1996 (received for review April 26, 1996)

ABSTRACT **A threonine to isoleucine polymorphism at amino acid 164 in the fourth transmembrane spanning domain of the β_2-adrenergic receptor (β_2AR) is known to occur in the human population. The functional consequences of this polymorphism to catecholamine signaling in relevant cells or to end-organ responsiveness, however, are not known. To explore potential differences between the two receptors, site-directed mutagenesis was carried out to mimic the polymorphism. Transgenic FVB/N mice were then created overexpressing wild-type (wt) β_2AR or the mutant Ile-164 receptor in a targeted manner in the heart using a murine α myosin heavy chain promoter. The functional properties of the two receptors were then assessed at the level of *in vitro* cardiac myocyte signaling and *in vivo* cardiac responses in intact animals. The expression levels of these receptors in the two lines chosen for study were ≈1200 fmol/mg protein in cardiac membranes, which represents a ≈45-fold increase in expression over endogenous βAR. Myocyte membrane adenylyl cyclase activity in the basal state was significantly lower in the Ile-164 mice (19.5 ± 2.7 pmol/min/mg) compared with wt β_2AR mice (35.0 ± 4.1 pmol/min/mg), as was the maximal isoproterenol-stimulated activity (49.8 ± 7.8 versus 77.1 ± 7.3 pmol/min/mg). In intact animals, resting heart rate (441 ± 21 versus 534 ± 17 bpm) and dP/dt_{max} (10,923 ± 730 versus 15,308 ± 471 mmHg/sec) were less in the Ile-164 mice as compared with wt β_2AR mice. Similarly, the physiologic responses to infused isoproterenol were notably less in the mutant expressing mice. Indeed, these values, as well as other contractile parameters, were indistinguishable between Ile-164 mice and nontransgenic littermates. Taken together, these results demonstrate that the Ile-164 polymorphism is substantially dysfunctional in a relevant target tissue, as indicated by depressed receptor coupling to adenylyl cyclase in myocardial membranes and impaired receptor mediated cardiac function *in vivo*. Under normal homeostatic conditions or in circumstances where sympathetic responses are compromised due to diseased states, such as heart failure, this impairment may have important pathophysiologic consequences.**

Recent studies have shown that the gene encoding the β_2-adrenergic receptor (β_2AR) displays a moderate degree of heterogeneity in the human population (1, 2). Four polymorphisms in the coding block of the gene result in amino acid changes from what has been considered the wild-type sequence (3) at positions 16, 27, 34, and 164. Using site-directed mutagenesis and recombinant expression in fibroblasts, the pharmacologic properties of these variants have been assessed (4, 5). The Ile-164 variant, where Ile has been substituted for Thr at position 164 in the fourth transmembrane spanning domain of the receptor, has been found to be substantially uncoupled from G_s in both the nonagonist and agonist occupied forms, when studied using such model cell systems. The relevance of the Ile-164 receptor to signal transduction in a pertinent cell such as the cardiac myocyte or its potential role in end-organ function are not known.

The human heart expresses both the β_1AR and β_2AR subtypes (6). Each receptor mediates positive inotropic and chronotropic responses to endogenous catecholamines and exogenously administered agonists (7). Most forms of congestive heart failure, including the "idiopathic" forms, are characterized by a diminished responsiveness to β-agonists (8). As a probable consequence of elevated catecholamines (and perhaps of other factors as well), β_1AR expression has been noted to be markedly reduced in patients with idiopathic dilated cardiomyopathy, a finding that has not been observed for the β_2AR (8). The β_2AR, then, may take on an even greater role in providing for cardiac responses to increased sympathetic drive or exogenous agonists under these circumstances. We have considered that if the Ile-164 variant is in fact dysfunctional in the heart, individuals harboring this receptor with heart failure may exhibit marked decompensation.

The current study was undertaken to explore the relevance of the Ile-164 receptor in cardiac myocyte signaling at both the cellular and the whole organ levels. The strategy was to express in transgenic mice the human wild-type β_2AR (wt β_2AR) or the Ile-164 variant in a cardiac specific manner using the murine α myosin heavy chain (αMHC) promoter. *In vitro* and *in vivo* assessments of receptor function were then undertaken that revealed a substantial impairment imposed by this polymorphism in cellular as well as intact heart function.

METHODS

Transgenic Mice. Cardiac-specific expression of the β_2AR in transgenic mice was achieved using the murine αMHC promoter (9). Briefly, the coding block of human wt β_2AR cDNA was blunt-end ligated into an αMHC promoter construct (9) at the *SalI* site. To generate the Ile-164 polymorphism, oligonucleotide-directed site-specific mutagenesis was carried out on the wild-type template, substituting thymidine for cytidine at nucleic acid 491 as described (4). This mutated cDNA was also cloned into the αMHC *SalI* site, and the integrity of the two constructs was verified by dideoxy sequencing. Each was then digested by *NotI* and the liberated fragment was then isolated, purified, and used for injection into male pronuclei of embryos

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviations: βAR, β-adrenergic receptor; wt β_2AR, wild-type βAR; αMHC, α myosin heavy chain; [^{125}I]CYP, [^{125}I]cyanopindolol; dP/dt, first derivative of left ventricular systolic pressure.

§To whom reprint requests should be addressed at: University of Cincinnati College of Medicine, 231 Bethesda Avenue, ML0564, Cincinnati, OH 45267-0564.

from FVB/N mice. These were implanted into oviducts of pseudopregnant females. Pups were screened for the presence of the transgene by use of the polymerase chain reaction performed on genomic DNA derived from ear clips using a sense primer based on the αMHC promoter sequence (5′-CACATAGAAGCCTAGCCCACA-3′) and an antisense primer based on the β_2AR coding block sequence (5′-CCAATTTAGGAGGATGTAAACTTC-3′). Founders that were positive for the transgene were mated with nontransgenic animals to establish eight lines heterozygous to the transgenes. Two lines expressing these two receptors at ≈1200 fmol/mg were propagated for further study. Second and third generation heterozygous mice that were 9–11 weeks of age and weighed 23–30 g were used for all studies. In this report, the transgenic mice are denoted as either "wt β_2AR" or "Ile-164" mice, whereas mice without transgene incorporation are denoted as "nontransgenic littermates."

***In Vivo* Cardiac Function.** Cardiac and systemic hemodynamics were assessed in mice using intact, spontaneously breathing, animals as described (10). The protocol was approved by the University of Cincinnati Institutional Animal Care and Use Committee. Briefly, animals were anesthetized to a surgical plane of anesthesia with a single dose of intraperitoneal ketamine (50 μg per g body weight) and thiobutabarbital (100–200 μg per g body weight). A tracheotomy was performed to maintain airway patency. Body temperature was monitored with a rectal probe and was maintained with a feedback-controlled heating table and heating lamp. For systemic blood pressure measurements, the right femoral artery was cannulated with the tip of the catheter advanced to a point just distal to the femoral takeoff. Pressures were recorded using a Cobe Laboratories (Arvada, CO) model CDXIII transducer. The right femoral vein was catheterized in a similar fashion and was used for infusion of drugs. For measuring left ventricular function, a catheter with a Millar Instruments (Houston) Mikro-Tip pressure transducer (600 μm diameter, 10,000 Hz frequency response) was advanced from the right carotid artery into the left ventricle. Placement was confirmed by appearance of characteristic ventricular pressure profiles and verified at the conclusion of the study by direct observation. As assessed by the arterial pressure and ventricular pressure waveforms, there was no evidence of aortic valve regurgitation due to the presence of the catheter. Similarly, gross and histologic examination of hearts studied in this manner showed no myocardial or aortic valve damage. Thirty minutes after instrumentation, baseline measurements were obtained, followed by measurements in response to infusions (0.1 μl per g body weight per min) of varying concentrations of isoproterenol. Six doses from 0.01–0.32 mg per g body weight per min were infused. Doses were administered over a 3-min period with responses being recorded over the last 30 sec of the infusion period. Subsequent doses were administered after contractile parameters recovered to baseline (≈10 min). Pressure signals from the Cobe Laboratories and Millar Instruments transducers were amplified using custom-made amplifiers and the output was recorded using MACLAB hardware and software (MACLAB 4/s, AD Instruments, Milford, MA) at a sampling rate of 1000 samples per sec. Signals for arterial pressure, intraventricular pressure, and ventricular dP/dt were analyzed off-line using the MACLAB analysis software. Responses were meaned over a 30-sec interval. Comparisons were primarily made between transgenic mice expressing wt β_2AR or the Ile-164 receptor. As indicated, some comparisons were also made with the responses of nontransgenic littermates. Data were analyzed using a mixed, two-factor ANOVA, with repeated measures on the second factor (dose). Post hoc comparisons were made using single degree-of-freedom comparison contrasts. All analyses were performed using SUPER ANOVA software by Abacus Concepts (Berkeley, CA).

Adenylyl Cyclase Assays. Mice were killed by CO_2 narcosis, and the hearts were extracted, placed in cold 5 mM Tris/2 mM EDTA (pH 7.4) buffer, and homogenized with a Brinkmann polytron (75% maximal setting for 15 sec). In this and in all subsequent steps buffers contained the protease inhibitors leupeptin (10 μg/ml), soybean trypsin inhibitor (5 μg/ml), benzamidine (10 μg/ml), and aprotinin (10 μg/ml). Particulates were then brought up in 10 vol of the same buffer and centrifuged at 4°C for 15 min at 40,000 × *g*. The crude membranes were resuspended a second time in 10 vol of buffer and centrifuged again as above. The pellet was then resuspended with the polytron for 2 sec in a buffer that provided for 100 mM Tris, 20 mM $MgCl_2$, and 2 mM EDTA (pH 7.4) in the assay. The resulting suspension was then filtered through nylon mesh and used for adenylyl cyclase and protein assays. For adenylyl cyclase assays, ≈150 μg of membranes were added to a reaction mixture containing 0.2 mM ATP, 0.1 mM GTP, 1.0 mM ascorbic acid, 1.0 mM isobutylmethylxanthine, 25 mM phosphorocreatine, and 1 mg/ml creatine phosphokinase, with 10 nM–100 μM isoproterenol or water (basal). Reactions were carried out for 5 min at 37°C and terminated by addition of HCl to 0.1 M. The tubes were then centrifuged at 1000 × *g* for 10 min, and an aliquot of the supernatant was assayed for cAMP using a radioimmunoassay as described (11). Protein was determined by the copper bicinchonicic acid method (12) using bovine serum albumin as standard.

Radioligand Binding. Membranes derived after the first centrifugation step from the procedure described above were used for radioligand binding with [^{125}I]cyanopindolol ([^{125}I]CYP) using methods essentially as described elsewhere (4, 13). Briefly, for competition studies, membranes were incubated with 30 pM of [^{125}I]CYP and various concentrations of agonists or antagonists for 2 hr at 25°C. As indicated, in some experiments the reactions also contained the nonhydrolyzable GTP analogue, $G_{pp}NH_p$ (100 μM). For determination of receptor expression (B_{max}), membranes were incubated with a saturating concentration of [^{125}I]CYP (400 pM) in the absence (total binding) or presence (nonspecific binding) of 10 μM propranolol. Reactions were terminated by dilution with cold 10 mM Tris (pH 7.4) buffer and vacuum filtration over glass fiber filters. Specific binding was defined as total minus nonspecific binding and was normalized to protein. Competition studies were analyzed as previously described (4) by iterative least squares techniques.

RESULTS

In the nontransgenic FVB/N mouse, cardiac membranes were found to express ≈26 fmol/mg of βAR as assessed by radioligand binding (Fig. 1). The transgenic strategy used resulted in a marked enhancement of βAR expression in the heart in the eight founder mice, ranging from 5- to 45-fold over nontransgenic littermates. Consistent with what has been reported by Milano *et al.* (9), who used this same targeting strategy, overexpression was detected in all four chambers of the heart, but no appreciable increase in [^{125}I]CYP binding was observed in vena cava, lung, aorta, or skeletal muscle. Two lines that stably expressed the wt β_2AR (1224 ± 176 fmol/mg) or the Ile-164 mutant (1151 ± 217 fmol/mg) at similar levels were used for further study (Fig. 1). In nontransgenic mice, the β_2AR expression represented ≈30% of all βAR as assessed in radioligand competition studies with the β_2AR-specific antagonist ICI118551 (data not shown). As expected, in transgenic animals, virtually the entire population of cardiac receptors was of the β_2AR subtype. As has been previously reported (9), cardiac-specific overexpression of β_2AR did not result in enhanced neonatal mortality, gross phenotypic abnormalities, or an increase in the incidence of death in adults as assessed up to 6 months of age. Likewise, overexpression of the Ile-164 receptor had no apparent deleterious effects. In agonist com-

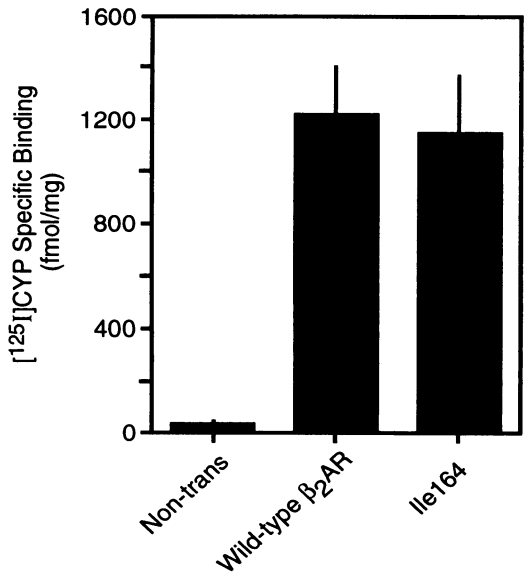

FIG. 1. Cardiac βAR expression in transgenic and nontransgenic mice. Myocardial membranes were prepared from a line of transgenic FVB/N mice overexpressing wt β_2AR or the Ile-164 variant or from nontransgenic littermates. Receptor expression was quantitated by radioligand binding with [^{125}I]CYP, using 10 μM propranolol to define nonspecific binding as described. Shown are results (mean ± SE) from five independent experiments performed with membranes from five mice from each group.

petition studies with cardiac membranes derived from these transgenic mice, the Ile-164 mutant displayed an affinity for isoproterenol that was similar to that found with wt β_2AR (380 ± 50 nM versus 490 ± 90 nM; $P > 0.05$, $n = 3$). A clear functional difference, however, was noted between the two receptors as assessed in myocardial membrane adenylyl cyclase assays (Fig. 2). Basal activities for the Ile-164 receptor were 19.5 ± 2.7 pmol/min/mg compared with 35.0 ± 4.1 pmol/

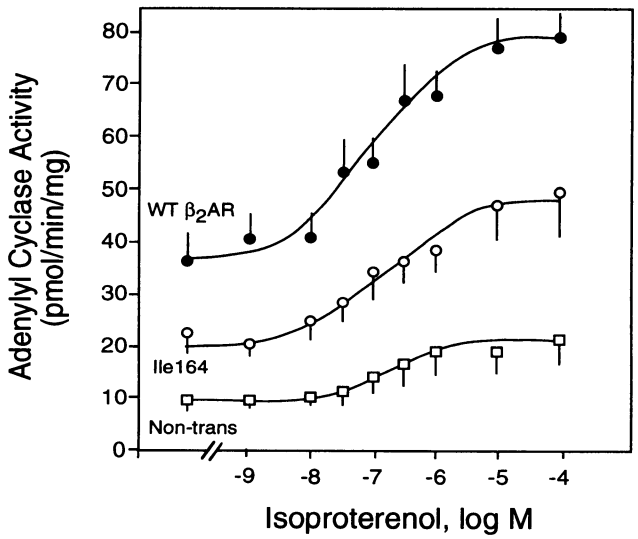

FIG. 2. Adenylyl cyclase activities of cardiac membranes derived from wt β_2AR or Ile-164-overexpressing transgenic mice. Membranes were prepared and adenylyl cyclase activities determined as described. Basal and maximal isoproterenol-stimulated activities of the Ile-164 receptor were lower than that of the wt β_2AR ($P < 0.01$), and the EC_{50} was ≈3-fold greater for Ile-164 as compared with the wt β_2AR (143.9 ± 23.4 nM versus 45.5 ± 7.4 nM; $P < 0.01$). Activities from nontransgenic littermates were lower than those from either transgenic line. Shown are the mean ± SE from independent experiments performed with membranes from six mice from each group.

min/mg for wt β_2AR ($P < 0.01$, $n = 6$). Maximal isoproterenol-stimulated activities were also depressed with the Ile-164 receptor (49.8 ± 7.8 pmol/min/mg) as compared with wild-type receptor (77.1 ± 7.3 pmol/min/ mg; $P < 0.01$, $n = 6$). The concentration of isoproterenol that resulted in a half-maximal response in these adenylyl cyclase assays (the EC_{50}) was ≈3-fold higher for the mutant as compared with wt β_2AR (143.9 ± 23.4 nM versus 45.5 ± 7.4 nM; $P < 0.01$, $n = 6$). These depressed adenylyl cyclase activities observed with the Ile-164 mice were nevertheless greater than those of nontransgenic littermates that had basal activities of 9.3 ± 1.9 pmol/min/mg, and maximal isoproterenol-stimulated activities of 20.5 ± 4.1 pmol/min/mg.

The *in vivo* cardiac responses to infused isoproterenol are shown in Figs. 3 and 4. Representative recordings of heart rate, left ventricular systolic pressure, and the first derivative of left ventricular systolic pressure (dP/dt) are shown in Fig. 3. Under the conditions used, the absolute values for left ventricular pressure, dP/dt_{max}, and dP/dt_{min} in the nontransgenic mice were ≈2-fold higher in magnitude than those reported by Rockman and colleagues (9, 14, 15). This difference is most likely the result of measurements being made in spontaneously breathing intact animals in our study, as compared with mechanically ventilated animals with open thorax and pericardium in the aforementioned studies. Nevertheless, the responses to isoproterenol in nontransgenic mice are quite similar with the two methods (Fig. 4).

Resting heart rates for the Ile-164 mutant mice were ≈100 bpm less than those of wt β_2AR expressing mice (441 ± 21 versus 534 ± 17 bpm; $n = 5$, $P < 0.001$). Interestingly, the resting heart rate of the mutant-expressing mice was not significantly different than that of nontransgenic littermates. In response to isoproterenol, the Ile-164 mice had lower heart rates as compared with wt β_2AR for all doses except for the highest (Fig. 4*A*). In fact, the heart rate responses in the Ile-164 mice were indistinguishable from those of nontransgenic mice. When the entirety of the heart rate responses was assessed, the pattern of change between wt β_2AR mice and Ile-164 mice differed significantly ($P = 0.008$). The Ile-164 mice displayed an overall response that was not different from that of nontransgenic littermates ($P = 0.8$). The changes in dP/dt_{max} in response to isoproterenol are shown in Fig. 4*B*. Resting dP/dt_{max} was depressed in the Ile-164 mice (10,923 ± 730 mmHg/sec) as compared with wt β_2AR expressing mice (15,308 ± 471 mmHg/sec; $n = 5$, $P < 0.005$). The resting dP/dt_{max} of the Ile-164 mice did trend toward being higher than that of nontransgenic littermates (8885 ± 630 mmHg/sec), but this did not reach statistical significance (Fig. 4*B*). At all but the two highest doses of infused isoproterenol, the dP/dt_{max} of the Ile-164 mice was depressed as compared with mice overexpressing wt β_2AR, and the overall response to isoproterenol significantly differed ($P < 0.05$) between the two groups. The dP/dt_{max} responses for the Ile-164 mice trended toward being greater than those of nontransgenic littermates, but these were not statistically different. The above differences in basal and isoproterenol-stimulated responses between Ile-164 and wt β_2AR-expressing transgenic mice were also observed with other indices of ventricular function. Lower resting left ventricular systolic pressures (103 ± 3 versus 112 ± 3 mmHg), left ventricular end diastolic pressures (4.2 ± 0.8 versus 9.7 ± 2.1 mmHg), and dP/dt_{min} (−9836 ± 445 versus −13,445 ± 555 mmHg/sec) were observed in the Ile-164 mice compared with the wt β_2AR mice ($P < 0.05$). With infusion of isoproterenol, the overall responses of these parameters differed significantly ($P < 0.05$) between the two groups, in patterns that paralleled those of the heart rate and dP/dt_{max} responses shown in Fig. 4.

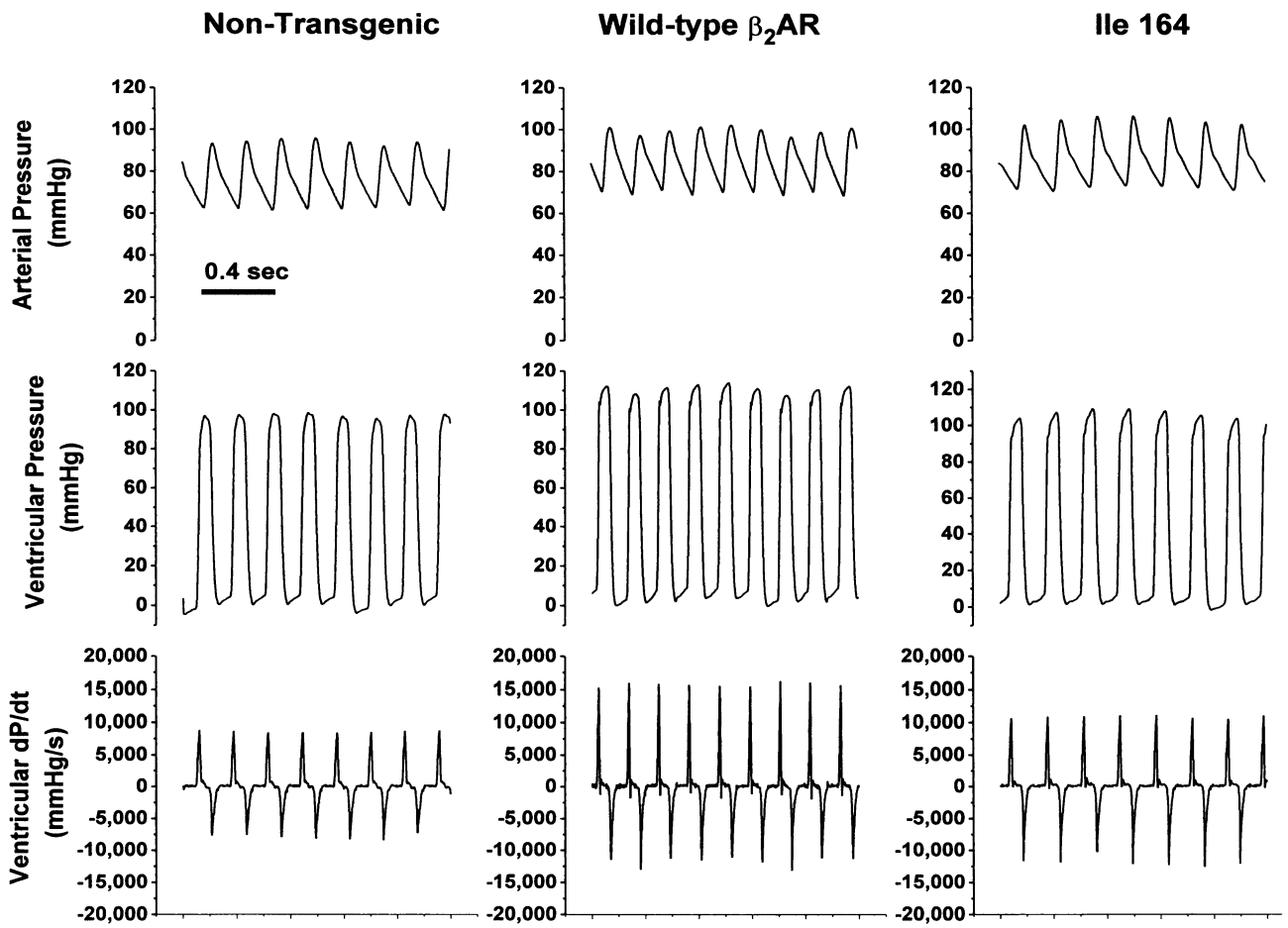

FIG. 3. *In vivo* hemodynamics of transgenic and nontransgenic mice. Shown are representative tracings of heart rate, left ventricular pressure, and the first derivative of left ventricular pressure (dP/dt) obtained in resting animals that were instrumented as described. Resting heart rate, left ventricular pressure, dP/dt_{max}, and dP/dt_{min} were greater in the wt β_2AR expressing mice as compared with the Ile-164 mice and nontransgenic littermates (see text). Selected responses to isoproterenol are provided in Fig. 4.

DISCUSSION

These studies were undertaken to assess the relevance of the Ile-164 β_2AR polymorphism to catecholamine-mediated myocardial signaling at the cellular and intact organ level. The isolated Ile-164 polymorphism has been detected in 6–8% of the population (2) and initial studies of the mutated recombinant receptor have suggested impaired function (4). Given the importance of β_2AR in mediating resting and catecholamine-stimulated inotropy and chronotropy in the heart, we have considered that this polymorphism may be an important factor in normal cardiovascular homeostasis or may represent an important disease modifier in conditions such as heart failure. While the β_1AR subtype is expressed to a greater extent in the human heart as compared with the β_2AR subtype, the latter has been shown to couple more efficiently to G_s in the human heart (6), and β_2AR-mediated increases in inotropy and chronotropy are well-documented (7). Such β_2AR responses may be particularly important in heart failure where β_1AR expression is markedly down-regulated (8).

To initially assess the pharmacologic consequences of the Thr to Ile substitution at position 164, we used site-directed mutagenesis of the wt β_2AR cDNA. This construct was subcloned into a mammalian expression vector and transfected into Chinese hamster fibroblasts (CHW-1102 cells). The mutated and wild-type receptors were studied at multiple levels of expression in these cells and several differences between the two were observed (4). The most dramatic was related to functional coupling of the receptor to G_s. In the absence of agonist, basal adenylyl cyclase activities were ≈2- to 3-fold lower in cells expressing the Ile-164 receptor as compared with wt β_2AR. Maximal agonist-stimulated activities were also depressed ≈50% with the Ile-164 receptor, and the EC_{50} for agonist mediated stimulation of adenylyl cyclase was increased ≈3-fold with the mutant receptor. In addition, the affinities of epinephrine, norepinephrine and isoproterenol for the Ile-164 receptor were 3- to 4-fold lower than those for wild type. We do not believe that this minimal decrease in agonist affinity (which was found in the recombinant study but not in the current study) is the basis for the defect with the mutant receptor because (*i*) the maximal agonist stimulation of adenylyl cyclase was depressed and (*ii*) basal (nonagonist) levels of receptor-mediated stimulation of adenylyl cyclase were also depressed. While the mechanism responsible for receptor dysfunction imparted by this mutation at position 164 is not entirely clear, we have hypothesized (4) that the critical function of nearby Ser-165 (16) is perturbed, resulting in conformational effects that alter both agonist and nonagonist coupling of the receptor to G_s. However, the importance of such impairment as observed using fibroblast cell lines to receptor function in a relevant cell or to global organ function could only be inferred since receptor function can be highly dependent on cell type. Indeed, β_1AR and β_2AR signaling in myocardial membranes differ in several aspects from that observed in recombinant cells (6, 11, 17). Thus, we felt that it was important to investigate the potential importance of this polymorphism in relevant cells, such as myocytes, and also to extend such findings to intact organ responsiveness.

The approach used to address these goals differs somewhat from that of others who have reported transgenic expression

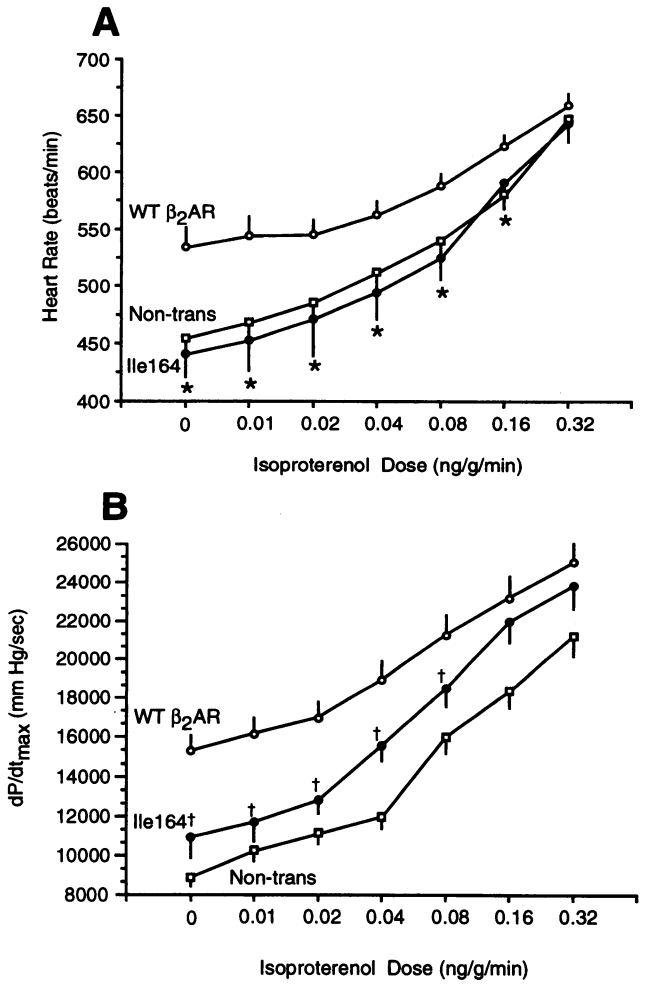

FIG. 4. *In vivo* cardiac responses to isoproterenol in transgenic and nontransgenic mice. Transgenic mice overexpressing wt β_2AR or the Ile-164 variant or nontransgenic littermates were instrumented as described and *in vivo* hemodynamics obtained in the resting state and during administration of six doses of isoproterenol. Resting and isoproterenol-stimulated heart rates were significantly less with the Ile-164 animals as compared with wt β_2AR mice except at the highest dose and the overall response between the two groups differed significantly ($P = 0.008$). Similar findings were observed with resting and isoproterenol-stimulated dP/dt_{max}. Statistical comparisons are between mice expressing wt β_2AR and Ile-164 mutant. *, $P < 0.001$; †, $P < 0.01$. The heart rate and dP/dt_{max} values for Ile-164 mice were not different than those of nontransgenic littermates. Shown are mean ± SE values from experiments performed with five mice from each line.

of the β_2AR in the heart (9, 14). In most of the studies reported by Milano *et al.* (9) and Bond *et al.* (14), wt human β_2AR was substantially overexpressed (≈200-fold over total βAR expressed in nontransgenic mice). This resulted in near maximal basal inotropic and chronotropic states of the heart, with very little responsiveness to isoproterenol. To address the relevance of the Ile-164 mutant, we aimed to express the wt β_2AR over background to a level that clearly distinguished cardiac function of the transgenic mice from nontransgenic littermates. Yet, we wanted a level of expression that still provided for dose-dependent *in vivo* responses to isoproterenol. As is shown in Fig. 4, expression of wt β_2AR at ≈1200 fmol/mg accomplished this objective. Baseline parameters of the wt β_2AR mice were notably different than those of nontransgenic mice, and heart rate and contractile relaxation responses were increased with infusion of isoproterenol. All these responses were greater than those of the nontransgenic mice except at the two highest doses of isoproterenol where they were similar. Mice expressing the Ile-164 receptor, also at ≈1200 fmol/mg, clearly displayed depressed basal and isoproterenol-stimulated cardiac responses as compared with wt β_2AR-expressing mice. Indeed, in the case of heart rate responses, the Ile-164 mice were indistinguishable from nontransgenic mice. With the other measurements, there were little discernible differences between Ile-164 mice and nontransgenic mice. We have interpreted these results as being consistent with the Ile-164 receptor being markedly impaired at the intact heart level.

The results of these physiologic studies are comparable with what was found with isolated cardiac membranes. Basal adenylyl cyclase levels were lower in Ile-164 receptor-bearing membranes, which is consistent with the observed depressed resting cardiac function in these mice. Isoproterenol-stimulated activities were also lower with the Ile-164 mutant as compared with wt β_2AR, and the dose-response curve for the mutant was shifted rightward. When the signal transduction efficiency of the two receptors in cardiac membranes is calculated using the transducer coefficient ("Tau") method of Black *et al.* (18), the mutant receptor is ≈80% impaired compared with wt β_2AR. This is consistent with the observed minimal difference in physiologic parameters between the Ile-164 mice and nontransgenic littermates. It is interesting to note that while the maximal agonist-stimulated adenylyl cyclase activity of the Ile-164 receptor was found to be significantly lower than that of wt β_2AR, the physiologic responses to the highest concentrations of agonist were similar. This implies that at a certain level of G_s stimulation in the heart a maximal response can be elicited, with the further stimulation that is observed *in vitro* having no physiologic significance. This notion is consistent with the results of Milano *et al.* (9). In these studies, agonist-induced stimulation of adenylyl cyclase over basal levels was in fact observed in transgenic mice substantially overexpressing the wt β_2AR, yet the *in vivo* physiologic responses were maximal at baseline and were not changed by infusion of agonist.

One potential limitation of the current study is that the wt β_2AR and the Ile-164 variant were expressed against a background of endogenous receptors. However, efforts to date to knock out β_2AR in mice via gene ablation techniques, which would provide for a null background, have not been successful. As such, our approach of modest expression of β_2AR over endogenous receptor levels did lead to an altered phenotype with the wild-type receptor. This was manifested as an enhanced resting and isoproterenol-stimulated cardiac function. However, the Ile-164 mutant receptor, despite being expressed to the same level and having equivalent affinity for agonist as the wt β_2AR overexpressor, exhibited a cardiac phenotype that was virtually indistinguishable from that of nontransgenic mice. Taken together, these results point toward the Ile-164 receptor variant being substantially impaired. In conditions where sympathetic responses are compromised due to diseased states such as congestive heart failure, such impairment may have important pathophysiologic consequences. Clinical studies to date with the more prevalent polymorphisms at amino acid positions 16 and 27, which differ in their down-regulation phenotypes, indeed support the concept that these polymorphic forms of the receptor may act as disease modifiers (2, 19–21). In addition, polymorphisms such as Ile-164 may be the basis of the well-established interindividual variation in catecholamine responsiveness observed in the general population (22).

In summary, we have mimicked a naturally occurring polymorphism of the β_2AR, Ile-164, found in the human population using site-directed mutagenesis, and created transgenic mice with cardiac-specific overexpression of this mutant or the wt β_2AR. The Ile-164 variant was markedly impaired as assessed by *in vitro* studies of receptor coupling to adenylyl cyclase in myocardial membranes and by *in vivo* studies of

cardiac function. This represents the first investigation of a naturally occurring mutation of a G protein coupled receptor in transgenic mice, and strongly supports the concept that the Ile-164 β_2AR variant is relevant to cardiac function in the intact animal.

This work was supported by National Institutes of Health Program of Excellence in Molecular Biology of Heart and Lung Grant HL41496.

1. Reihsaus, E., Innis, M., MacIntyre, N. & Liggett, S. B. (1993) *Am. J. Resp. Cell Mol. Biol.* **8,** 334–339.
2. Liggett, S. B. (1995) *News Physiol. Sci.* **10,** 265–273.
3. Kobilka, B. K., Dixon, R. A., Frielle, T., Dohlman, H. G., Bolanowski, M. A., Sigal, I. S., Yang-Feng, T. L., Krancke, U., Caron, M. G. & Lefkowitz, R. J. (1987) *Proc. Natl. Acad. Sci. USA* **84,** 46–50.
4. Green, S. A., Cole, G., Jacinto, M., Innis, M. & Liggett, S. B. (1993) *J. Biol. Chem.* **268,** 23116–23121.
5. Green, S., Turki, J., Innis, M. & Liggett, S. B. (1994) *Biochemistry* **33,** 9414–9419.
6. Bristow, M. R., Hershberger, R. E., Port, J. D., Minobe, W. & Rasmussen, R. (1988) *Mol. Pharmacol.* **35,** 295–303.
7. Brodde, E. O., Schuler, S., Kretsch, R., Brinkmann, M., Borst, H. G., Hetzer, R., Reidemeister, J. C., Warnecke, H. & Zerkowski, H. R. (1986) *J. Cardiovasc. Pharmacol.* **8,** 1235–1242.
8. Bristow, M. R., Ginsberg, R., Umans, V., Fowler, M., Minobe, W., Rasmusen, R., Zera, P., Menlove, R., Shah, P., Jamieson, S. & Stinson, E. B. (1986) *Circ. Res.* **59,** 297–309.
9. Milano, C. A., Allen, L. F., Rockman, H. A., Dolber, P. C., McMinn, T. R., Chien, K. R., Johnson, T. D., Bond, R. A. & Lefkowitz, R. J. (1994) *Science* **264,** 582–586.
10. Lorenz, J. N. & Kranias, E. (1995) *Circulation* **92,** I657–I658 (abstr.).
11. Green, S. & Liggett, S. B. (1994) *J. Biol. Chem.* **269,** 26215–26219.
12. Smith, P. K., Krohn, R. I., Hermanson, G. T., Mallia, A. K., Gartner, F. H., Provenzano, M. D., Fujimoto, E. K., Goeke, N. M., Olson, B. J. & Klenk, D. C. (1985) *Anal. Biochem.* **150,** 76–85.
13. Schwinn, D. A., Leone, B., Spann, D. R., Chesnut, L. C., Page, S. O., McRay, R. L. & Liggett, S. B. (1991) *Circulation* **84,** 2559–2567.
14. Bond, R. A., Leff, P., Johnson, T. D., Milano, C. A., Rockman, H. A., McMinn, T. R., Apparsundaram, S., Hyek, M. F., Kenakin, T. P., Allen, L. F. & Lefkowitz, R. J. (1995) *Nature (London)* **374,** 272–275.
15. Koch, W. J., Rockman, H. A., Samama, P., Hamilton, R. A., Bond, R. A., Milano, C. A. & Lefkowitz, R. J. (1995) *Science* **268,** 1350–1353.
16. Strader, C. D., Candelore, M. R., Hill, W. S., Sigal, I. S. & Dixon, R. A. F. (1989) *J. Biol. Chem.* **264,** 13572–13578.
17. Green, S. A., Holt, B. D. & Liggett, S. B. (1992) *Mol. Pharmacol.* **41,** 889–893.
18. Black, J. W., Leff, P. & Shankley, N. P. (1985) *Br. J. Pharmacol.* **84,** 561–571.
19. Turki, J., Pak, J., Green, S., Martin, R. & Liggett, S. B. (1995) *J. Clin. Invest.* **95,** 1635–1641.
20. Hall, I. P., Wheatley, A., Wilding, P. & Liggett, S. B. (1995) *Lancet* **345,** 1213–1214.
21. Liggett, S. B. (1996) in *The Genetics of Asthma*, eds. Liggett, S. B. & Meyer, D. A. (Dekker, New York), pp. 455–478.
22. Liggett, S. B. & Raymond, J. R. (1993) in *Catecholamines*, ed. Bouloux, P. M. (Saunders, London), pp. 279–306.