

## Targeted Disruption of the $\beta 2$ Adrenergic Receptor Gene\*

(Received for publication, November 24, 1998, and in revised form, March 16, 1999)

Andrzej J. Chruscinski<sup>‡§</sup>, Daniel K. Rohrer<sup>¶</sup>, Eric Schauble<sup>||</sup>, Kavin H. Desai<sup>||</sup>, Daniel Bernstein<sup>||</sup>,  
and Brian K. Kobilka<sup>‡\*\*†§§</sup>

From the <sup>‡</sup>Department of Molecular and Cellular Physiology, <sup>\*\*</sup>Division of Cardiovascular Medicine, <sup>||</sup>Department of Pediatrics, the <sup>‡‡</sup>Howard Hughes Medical Institute, Stanford University, Stanford, California 94305 and the <sup>¶¶</sup>Department of Molecular Pharmacology, Roche Bioscience, Palo Alto, California 94304

**$\beta$ -Adrenergic receptors ( $\beta$ -ARs) are members of the superfamily of G-protein-coupled receptors that mediate the effects of catecholamines in the sympathetic nervous system. Three distinct  $\beta$ -AR subtypes have been identified ( $\beta 1$ -AR,  $\beta 2$ -AR, and  $\beta 3$ -AR). In order to define further the role of the different  $\beta$ -AR subtypes, we have used gene targeting to inactivate selectively the  $\beta 2$ -AR gene in mice. Based on intercrosses of heterozygous knockout ( $\beta 2$ -AR  $+/-$ ) mice, there is no prenatal lethality associated with this mutation. Adult knockout mice ( $\beta 2$ -AR  $-/-$ ) appear grossly normal and are fertile. Their resting heart rate and blood pressure are normal, and they have a normal chronotropic response to the  $\beta$ -AR agonist isoproterenol. The hypotensive response to isoproterenol, however, is significantly blunted compared with wild type mice. Despite this defect in vasodilation,  $\beta 2$ -AR  $-/-$  mice can still exercise normally and actually have a greater total exercise capacity than wild type mice. At comparable workloads,  $\beta 2$ -AR  $-/-$  mice had a lower respiratory exchange ratio than wild type mice suggesting a difference in energy metabolism.  $\beta 2$ -AR  $-/-$  mice become hypertensive during exercise and exhibit a greater hypertensive response to epinephrine compared with wild type mice. In summary, the primary physiologic consequences of the  $\beta 2$ -AR gene disruption are observed only during the stress of exercise and are the result of alterations in both vascular tone and energy metabolism.**

$\beta$ -Adrenergic receptors ( $\beta$ -ARs)<sup>1</sup> are members of the superfamily of G-protein-coupled receptors that are stimulated by the naturally occurring catecholamines, epinephrine and norepinephrine. As part of the sympathetic nervous system,  $\beta$ -ARs have been shown to have important roles in cardiovascular, respiratory, metabolic, central nervous system, and reproductive functions. Using techniques of molecular cloning, three distinct  $\beta$ -AR subtypes have been identified ( $\beta 1$ -AR,  $\beta 2$ -AR, and  $\beta 3$ -AR) (1–3). All three of these  $\beta$ -AR subtypes are believed to signal by coupling to the stimulatory G-protein  $G_s\alpha$  leading to activation of adenylyl cyclase and accumulation of the second messenger cAMP (1–3).

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

§ Supported in part by Medical Scientist Training Program Training Grant GM07365 from the NIGMS, National Institutes of Health.

§§ To whom correspondence should be addressed: Howard Hughes Medical Institute, Stanford University, Stanford, CA 94305. Tel.: 650-723-7069; Fax: 650-498-5092; E-mail: kobilka@cmgm.stanford.edu.

<sup>1</sup> The abbreviations used are:  $\beta$ -AR(s),  $\beta$ -adrenergic receptor(s); RER, respiratory exchange ratio; kb, kilobase pair; ES, embryonic stem; FFA, free fatty acid; [<sup>25</sup>I]-CYP, [<sup>125</sup>I]iodocyanopindolol.

Because of the diverse physiological functions mediated by  $\beta$ -ARs, much effort has been spent in understanding the roles of individual  $\beta$ -AR subtypes. In the past, researchers have relied on pharmacological tools such as subtype-selective agonists and antagonists to probe the function of the different  $\beta$ -AR subtypes. The presence of multiple  $\beta$ -AR subtypes was first suggested by Lands and co-workers (4, 5) who divided  $\beta$ -ARs into  $\beta 1$ -ARs and  $\beta 2$ -ARs. According to Lands' classification,  $\beta 1$ -ARs mediate cardiac stimulation, and  $\beta 2$ -ARs mediate smooth muscle relaxation in the peripheral vasculature and respiratory system. The presence of a third  $\beta$ -AR subtype was suggested when some of the effects of  $\beta$ -AR agonists could not be efficiently blocked by typical  $\beta$ -AR antagonists. This third  $\beta$ -AR subtype is now known as the  $\beta 3$ -AR and has been shown to have important roles in adipose tissue and the gastrointestinal tract (6).

Although both  $\beta 1$ -ARs and  $\beta 2$ -ARs are expressed in the heart of most mammalian species,  $\beta 1$ -ARs are expressed at higher levels and are recognized as playing the major role in regulating cardiac function. Functional studies have confirmed that activation of  $\beta 1$ -ARs leads to increased heart rate and force of contraction (7). Although they represent a smaller population in the heart than  $\beta 1$ -ARs,  $\beta 2$ -ARs have also been shown to play a role in regulating cardiac function in a variety of species (7–9). In studies using subtype-selective agonists and antagonists in the human heart,  $\beta 2$ -AR stimulation leads to activation of adenylyl cyclase and contributes to both inotropic and chronotropic responses (7). In the murine heart, however,  $\beta 2$ -ARs do not appear to couple to inotropic or chronotropic responses. When isolated cardiac muscle from  $\beta 1$ -AR knockout mice is stimulated with the non-subtype-selective  $\beta$ -AR agonist isoproterenol, neither inotropic nor chronotropic responses are observed (10).

In addition to their roles in the heart,  $\beta$ -ARs also regulate peripheral vascular tone. Stimulation of peripheral  $\beta$ -ARs leads to relaxation of vascular smooth muscle, thereby controlling the distribution of blood flow to different tissues. During exercise, for example, stimulation of  $\beta$ -ARs contributes to the increased blood flow to skeletal muscle. Based on the studies of Lands and co-workers (4, 5), the  $\beta$ -AR in the peripheral vasculature have been classified as the  $\beta 2$ -AR. Some reports, however, have shown roles for the other  $\beta$ -AR subtypes,  $\beta 1$ -ARs and  $\beta 3$ -ARs, in the peripheral vasculature (11–13).

Although much has been learned about the role of individual  $\beta$ -AR subtypes using classical pharmacological techniques, these studies are complicated by the fact that subtype-selective ligands are never perfectly selective. Moreover, at the doses required to block  $\beta$ -ARs *in vivo*, most  $\beta$ -AR ligands lose much of their subtype selectivity and may bind to other G-protein-coupled receptors such as serotonin receptors and dopamine receptors. Studies with  $\beta$ -AR ligands are especially difficult to interpret *in vivo* where it is hard to estimate the concentration

of ligands and their metabolites in target tissues. In order to further investigate the roles of the different  $\beta$ -AR subtypes in physiology, we have selectively inactivated the  $\beta$ 2-AR gene in mice using gene-targeting techniques. The knockout ( $\beta$ 2-AR  $-/-$ ) mice appear grossly normal and are fertile. Resting cardiovascular physiology is remarkably unperturbed in  $\beta$ 2-AR  $-/-$  mice. The major effects of  $\beta$ 2-AR gene disruption were observed only during the stress of exercise.  $\beta$ 2-AR  $-/-$  mice were able to exercise farther and with a lower respiratory exchange ratio at any given workload than wild type controls. However, they are hypertensive during exercise, suggesting an imbalance between the vasoconstrictive and vasorelaxant effects of endogenous catecholamines.

#### MATERIALS AND METHODS

**Targeting Vector Construction**—The targeting vector was constructed using sequence that had been cloned from a C57BL/6 mouse genomic library (14). In total, the targeting vector contained 11.4 kb of homology to the endogenous  $\beta$ 2-AR genomic locus. The gene for the  $\beta$ 2-AR was disrupted in the targeting vector by placing a neomycin (neo) resistance gene cassette into the coding sequence at a unique *Cl*aI site (15). This insertion disrupts the  $\beta$ 2-AR at the end of the fourth transmembrane segment and should produce a nonfunctional receptor. The short arm of the targeting vector was a 2.6-kb fragment from a 5' *E*coRI site to the *Cl*aI in the receptor. The long arm of the targeting vector (8.8 kb) extended from the *Cl*aI site in the receptor to a downstream *S*alI site. Also included in the vector was the herpes simplex virus thymidine kinase cassette to allow for negative selection when isolating ES cell clones (15). In order to screen for homologous recombinants a 5' external probe was used. This probe is a 300-base pair *B*amHI/*E*coRI fragment that detects a 4.9-kb fragment after mouse genomic DNA is digested with *B*amHI and then subjected to Southern blot analysis. In cases where the targeting vector has homologously recombined with the endogenous locus, the same probe would detect an additional band at 6.6 kb.

**Transfection of ES Cells**—R1 embryonic stem (ES) (16) cells were transfected using standard techniques (17). ES cells were grown on a monolayer of mouse embryonic fibroblasts in Dulbecco's modified Eagle's medium (UCSF tissue culture facility, San Francisco) supplemented with 20% fetal bovine serum (HyClone, Logan, UT), 1 mM sodium pyruvate (Life Technologies, Inc.), non-essential amino acids, and penicillin/streptomycin (UCSF Cell Culture Facility, San Francisco, CA),  $10^{-4}$  M  $\beta$ -mercaptoethanol (Specialty Media, Lavallette, NJ), and 2,000 units/ml of leukemia inhibitory factor (ESGRO; Life Technologies, Inc.). Cells were grown in an incubator at 37 °C in 95% air, 5% CO<sub>2</sub>. For the transfection, a 10-cm<sup>2</sup> dish of ES cells was transfected via electroporation with 20  $\mu$ g of targeting vector previously linearized with *Not*I. After selecting ES cells for 9 days in media containing G418 (Life Technologies, Inc.) and gancyclovir (Syntex, Palo Alto, CA), individual clones were picked and subcloned in 96-well plates. *B*amHI-digested DNA from clones was analyzed by Southern blot analysis with the 5' external probe. Nine homologous recombinants were isolated from 300 ES cell clones. Homologous recombinants were also screened with a neo probe to confirm that a single integration of the targeting vector had occurred.

**Morula Aggregation**—Chimeric mice were generated using the morula aggregation technique described previously (18). Briefly, embryos at morula stage (2.5 days pc) were isolated from oviducts of superovulated CD-1 mice by flushing the oviducts with M2 medium (Specialty Media, Lavallette, NJ). After removing the zona pellucida with an acidic Tyrode's solution (Specialty Media, Lavallette, NJ), the embryos were placed in depressions in a 6-cm tissue culture dish and covered with a droplet of M16 medium (Specialty Media, Lavallette, NJ). A protective layer of mineral oil (Sigma) was placed over the droplets. Clumps of ES cells with the targeted disruption (10–20 cells) were then seeded into the depression and placed in contact with the embryos. After an overnight incubation at 37 °C in 95% air, 5% CO<sub>2</sub>, the chimeric embryos were transferred to the uteri of pseudopregnant CD-1 hosts (20–25 embryos per host). Chimeric mice were identified in the resulting offspring by the presence of dark coat color patches. Chimeric males were then mated to FVB/N female mice to screen for germ line transmission of the ES cell DNA. After achieving germ line transmission,  $\beta$ 2-AR  $+/-$  mice were intercrossed to generate  $\beta$ 2-AR  $+/+$  and  $-/-$  mice for use in binding studies. For *in vivo* studies, the knockout allele was placed on a FVB/N background by backcrossing  $\beta$ 2-AR  $+/-$  mice to wild type FVB/N mice for four additional generations (5 back-

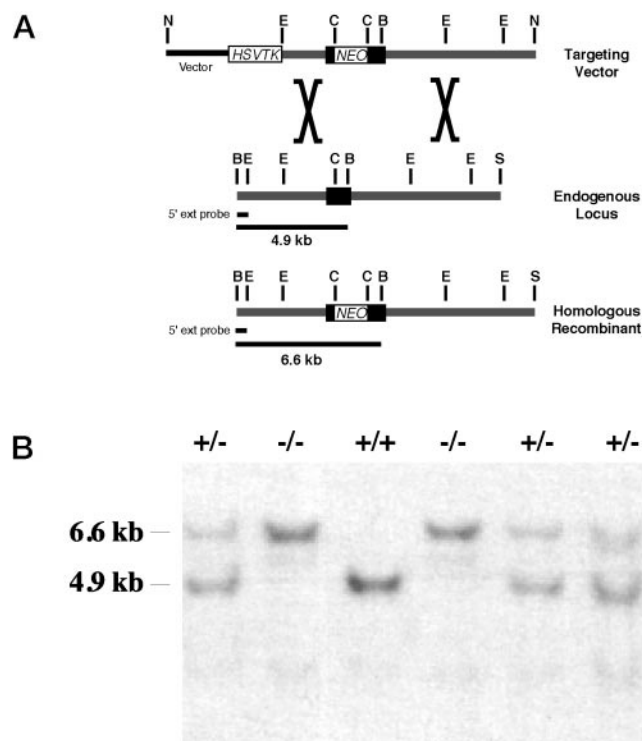
crosses to FVB/N in total).

**Binding Assays**—Whole lungs were dissected from wild type and knockout littermates, placed in a lysis buffer (10 mM Tris-HCl, 1 mM EDTA, pH 7.4), and homogenized with a Polytron (4  $\times$  20-s bursts). The membrane fraction was isolated by centrifugation at 10,000  $\times$  g and resuspended in binding buffer (75 mM Tris-HCl, 12.5 mM MgCl<sub>2</sub>, 1 mM EDTA, pH 7.4). Binding reactions were carried out by incubating membranes with the radioligand [<sup>125</sup>I]iodocyanopindolol (<sup>125</sup>I-CYP) (NEN Life Science Products) in 500- $\mu$ l volumes. After a 2-h incubation at room temperature, vacuum filtration was performed, and the filters were counted in a gamma counter. For saturation experiments, 3  $\mu$ g of membrane protein was incubated with increasing amounts of <sup>125</sup>I-CYP (1–300 pM). Nonspecific binding was determined in the presence of 1  $\mu$ M DL-propranolol (Sigma). For competition experiments, binding reactions were set up with 50 pM <sup>125</sup>I-CYP, 3–6  $\mu$ g of membrane protein, and varying concentrations (50 pM–13  $\mu$ M) of the  $\beta$ 2-AR-selective antagonist ICI 118,551 (Tocris Cookson, Ballwin, MO). Saturation and competition data were analyzed with GraphPAD software (GraphPAD Software Inc., San Diego, CA).

**In Vivo Cardiovascular Physiology**—*In vivo* studies were carried out as described previously (19). Adult male mice (12–16 weeks of age) were anesthetized with isoflurane using a vaporizer (Airoco Inc., Madison, WI), and a stretched Intramedic PE10 polyethylene catheter (Clay Adams, Parsippany, NJ) was inserted into the left carotid artery. The catheter was tunneled through the neck and then placed in a subcutaneous pouch in the back. After a minimum of 16 h recovery, the saline-filled catheter was removed from the pouch and connected to a Spectramed DTX Plus pressure transducer (Spectramed, Oxnard, CA). Output from the pressure transducer was amplified using a Gould 8-channel recorder and digitized using a Data Translation Series DT2801 analog-digital converter (Marlboro, MA). The digital signal was analyzed using Crystal Biotech Dataflow data acquisition software (Crystal Biotech, Hopkinton, MA) on a Gateway 2000 486DX2 microcomputer (Sioux City, SD). Baseline heart rate and mean arterial blood pressure were recorded after a 1-h equilibration period when the animals were awake but not active. In order to examine drug responses, drugs were administered through the carotid artery catheter. (–)-Isoproterenol hydrochloride (3  $\mu$ g/kg) and epinephrine bitartrate (3  $\mu$ g/kg) were purchased from Sigma and dissolved in saline for injection. In order to measure heart rate and blood pressure during exercise, cannulated mice were challenged with a graded treadmill exercise protocol (19) on a Simplex II rodent treadmill (Columbus Instruments, Columbus, OH). Treadmill activity was initiated at 3.5 m/min, 0° inclination, and increased to 5 m/min, 2° inclination 3 min later. Treadmill speed and inclination were then increased by 2.5 m/min and 2° inclination every 3 min thereafter. Exercise was terminated after the mice had completed 3 min at 20 m/min, 14° inclination. Mice that failed to complete the exercise protocol were excluded from the study.

**In Vivo Metabolic Responses to Exercise and Total Exercise Capacity**—In order to measure metabolic responses to exercise and exercise capacity, non-instrumented mice were challenged with the graded treadmill exercise protocol described above. Treadmill activity was initiated after the mice had equilibrated in the exercise chamber for 30–60 min. During the exercise protocol, oxygen consumption and carbon dioxide production were continuously monitored with an Oxyxam gas analyzer (Columbus Instruments, Columbus, OH). Stepwise increases in treadmill speed and inclination were made every 3 min until the mice stopped running from exhaustion. Exercise capacity was calculated as the total distance run by the animals during the exercise protocol.

**Body Weight, Epididymal Fat Pad Weight, Density, FFA Levels, and Glycerol Levels**—Male mice, 12–13 weeks old, were used for these studies. Mice were maintained in 12-h light/dark cycles. On the day of study, food was removed from the cage at the beginning of the light cycle, and mice were studied 3–5 h later. Each mouse was weighed and then anesthetized with 5% isoflurane for 45 s in an anesthesia induction box. The mouse was quickly removed from the box, and blood was collected by cardiac puncture with a 22-gauge needle. The mouse was then sacrificed via cervical dislocation. The volume of the mouse was determined by attaching a weight to the mouse and measuring the water displacement. Density was calculated as the body weight divided by the volume. After the volume measurement, both epididymal fat pads were dissected from the animal and weighed. The proportional weight of the fat pads was calculated by dividing the fat pad weight by the total body weight. After the blood samples had clotted in serum separator tubes (Becton Dickinson, Franklin Lakes, NJ), the samples were spun at 17,000  $\times$  g for 5 min to isolate the serum. Free fatty acid levels were determined with an enzymatic colorimetric kit (Wako Chemicals, Germany). Glycerol levels were determined with an enzy-



**FIG. 1. Gene targeting strategy for the  $\beta 2$ -AR gene.** *A*, shown from the top is the targeting vector, the endogenous locus of the  $\beta 2$ -AR gene, and the result of homologous recombination. *B*, *Bam*HI site; *C*, *Cla*I site; *E*, *Eco*RI site; *N*, *Not*I site; *S*, *Sal*I site; *Neo*, neomycin resistance cassette; *HSVTK*, thymidine kinase cassette. The black box represents coding sequence of  $\beta 2$ -AR gene, and the gray line represents untranslated genomic sequence. The 5' external probe and the expected fragments from Southern blot analysis are also shown. *B*, Southern blot of tail DNA from offspring of a heterozygous knockout intercross. Wild type (+/+), heterozygous knockout (+/-), and homozygous knockout (-/-) mice are recovered from this mating.

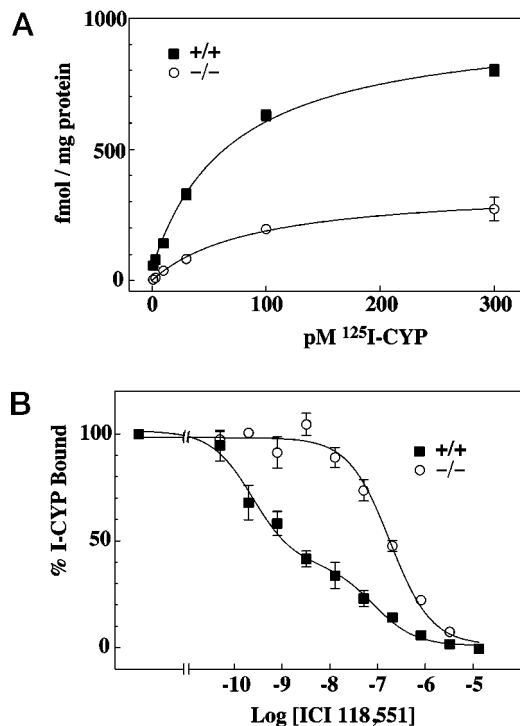
matic colorimetric kit (Roche Molecular Biochemicals).

**Locomotor Activity**—Locomotor activity of male mice, 12–13 weeks old, was measured by a photobeam cage system (San Diego Instruments, San Diego, CA). Mice were studied in pairs with a  $\beta 2$ -AR +/+ mouse and a  $\beta 2$ -AR -/- mouse placed in individual cages (30 × 50 cm). A frame containing 4 × 6 infrared photobeams was placed around each cage. Mice were placed in the cages at 5 p.m., and their activity was monitored as the number of beam breaks in a 48-h period. The mice used for these studies had not been used for any previous experiments.

## RESULTS

**$\beta 2$ -AR Gene Targeting**—Using standard ES cell techniques, the R1 ES cell line was transfected with the  $\beta 2$ -AR targeting vector shown in Fig. 1*A*. Homologous recombinants were identified by performing Southern blot analysis using the 5' external probe. Targeted clones were rescreened with a probe to the neomycin resistance gene to ensure that a single integration of the targeting vector had occurred (data not shown). Chimeric mice were generated with the targeted ES clones using the morula aggregation technique. Following germ line transmission of the knockout allele, heterozygous knockout pairs were intercrossed to generate  $\beta 2$ -AR +/+,  $\beta 2$ -AR +/-, and  $\beta 2$ -AR -/- mice. Shown in Fig. 1*B* is a Southern blot using DNA from the offspring of a  $\beta 2$ -AR +/- intercross.

**Results of Intercrosses**—After backcrossing  $\beta 2$ -AR +/- mice to wild type FVB/N mice for 5 generations,  $\beta 2$ -AR +/- mice were intercrossed. From 171 intercross progeny screened at weaning, 36  $\beta 2$ -AR +/+ mice, 91  $\beta 2$ -AR +/- mice, and 44  $\beta 2$ -AR -/- mice were identified. These results are consistent with the ratio predicted by Mendelian genetics ( $\chi$ -squared = 1.45,  $p > 0.4$ ). Thus, there is no embryonic or postnatal lethal-



**FIG. 2. Pharmacological characterization of  $\beta 2$ -AR +/+ and -/- mice.** *A*, saturation binding on membranes prepared from lungs of  $\beta 2$ -AR +/+ and -/- mice. Studies were performed by incubating membranes with varying concentrations of the  $\beta$ -AR antagonist  $^{125}$ I-CYP. Nonspecific binding was determined in the presence of 1  $\mu$ M DL-propranolol and subtracted from total binding. Values shown represent the mean  $\pm$  S.E. for three individual experiments. *B*, competition binding on membranes prepared from lungs of  $\beta 2$ -AR +/+ and -/- mice. Experiments were performed by incubating 3–6  $\mu$ g of membranes with 50 pM  $^{125}$ I-CYP and varying concentrations of the  $\beta 2$ -AR selective antagonist ICI 118,551. Nonspecific binding was determined in the presence of 1  $\mu$ M DL-propranolol. Values shown represent the mean  $\pm$  S.E. for three individual experiments.

ity associated with disruption of the  $\beta 2$ -AR gene in mice. After maturing into adults,  $\beta 2$ -AR -/- mice appear grossly normal and do not exhibit overtly abnormal behavior. Both  $\beta 2$ -AR -/- males and females are fertile.

**$\beta 2$ -AR Expression and Pharmacology in  $\beta 2$ -AR -/- Mice**—In order to verify that the genetic modification prevents expression of the  $\beta 2$ -AR gene, ligand binding experiments were performed using lung tissue isolated from  $\beta 2$ -AR +/+ and -/- littermates. Saturation binding experiments with the radioligand [ $^{125}$ I]iodocyanopindolol ( $^{125}$ I-CYP) demonstrate a reduction in total binding in the  $\beta 2$ -AR -/- mice (Fig. 2*A*). The  $B_{max}$  is reduced from 990 fmol/mg in  $\beta 2$ -AR +/+ mice to 360 fmol/mg in the  $\beta 2$ -AR -/- mice (36% of the wild type value). Competition binding experiments were performed using the  $\beta 2$ -AR-selective antagonist ICI 118,551 to characterize the residual  $^{125}$ I-CYP binding in the  $\beta 2$ -AR -/- mice (Fig. 2*B*). In lung membranes from wild type mice, the data were best fit by a biphasic curve with 62% high affinity ICI 118,551 binding ( $\beta 2$ -AR) sites and 38% low affinity ( $\beta 1$ -AR) sites. The competition binding data from  $\beta 2$ -AR -/- mice were best fit with a one-site curve that has low affinity for ICI 118,551. Thus, the residual  $^{125}$ I-CYP-binding sites in  $\beta 2$ -AR -/- lung are due to  $\beta 1$ -ARs, confirming the loss of  $\beta 2$ -AR-binding sites in  $\beta 2$ -AR -/- mice. These data also demonstrate that there has not been a compensatory change in  $\beta 1$ -AR expression in the lung as a result of the  $\beta 2$ -AR gene disruption;  $\beta 1$ -AR expression in  $\beta 2$ -AR -/- mice is 330 fmol/mg protein, whereas in  $\beta 2$ -AR +/+ mice,  $\beta 1$ -AR expression is 380 fmol/mg protein (0.38 × 990 fmol/mg protein).

TABLE I

Cardiovascular indices at rest and changes in cardiovascular indices after isoproterenol (Iso) and epinephrine (Epi) administration in  $\beta 2$ -AR +/+ and -/- mice

Values shown represent the mean  $\pm$  S.E. The number of mice studied is shown in parentheses. The isoproterenol-stimulated values represent the maximum changes in blood pressure (BP) and heart rate (HR) after drug administration. The epinephrine-stimulated values represent the maximum blood pressure change and the corresponding heart rate change after drug administration. The unpaired *t* test was used for statistical comparison between groups.

Genotype	Basal HR	Basal BP	$\Delta$ HR Iso stimulated	$\Delta$ BP Iso stimulated	$\Delta$ HR Epi stimulated	$\Delta$ BP Epi stimulated
	<i>bpm</i>	<i>mm Hg</i>	<i>bpm</i>	<i>mm Hg</i>	<i>bpm</i>	<i>mm Hg</i>
$\beta 2$ -AR +/+	641 $\pm$ 20 (10)	113.5 $\pm$ 2.4 (10)	141 $\pm$ 40 (7)	23.6 $\pm$ 1.7 (7)	19 $\pm$ 32 (7)	13.2 $\pm$ 1.9 (7)
$\beta 2$ -AR -/-	602 $\pm$ 42 (9)	114.2 $\pm$ 1.9 (9)	163 $\pm$ 56 (7)	12.6 <sup>a</sup> $\pm$ 1.9 (7)	-22 $\pm$ 17 (7)	27.1 <sup>b</sup> $\pm$ 3.2 (7)

<sup>a</sup> Significance at *p* = 0.0009 for comparing  $\beta 2$ -AR +/+ and -/- mice.

<sup>b</sup> Significance at *p* = 0.0027 for comparing  $\beta 2$ -AR +/+ and -/- mice.

**Cardiovascular Physiology**—In order to examine the effects of the gene disruption on whole animal physiology,  $\beta 2$ -AR +/+ and  $\beta 2$ -AR -/- mice were instrumented with carotid catheters to allow measurements of mean arterial blood pressure and heart rate in awake, non-anesthetized, and non-restrained mice. Under baseline resting conditions, mean blood pressure and heart rate were not significantly different between  $\beta 2$ -AR +/+ and  $\beta 2$ -AR -/- mice (Table I). In order to examine the effects of  $\beta$ -AR stimulation,  $\beta 2$ -AR +/+ and  $\beta 2$ -AR -/- mice were given an intra-arterial bolus of 3  $\mu$ g/kg of the non-selective  $\beta$ -AR agonist isoproterenol, a dose previously shown to produce maximal increases in heart rate and maximal reductions in blood pressure in wild type mice. Fig. 3 shows the typical response of a  $\beta 2$ -AR +/+ and a  $\beta 2$ -AR -/- mouse to isoproterenol. In  $\beta 2$ -AR +/+ mice, isoproterenol produced a rapid onset tachycardia and hypotension. In  $\beta 2$ -AR -/- mice, the tachycardic response to isoproterenol was preserved, but the hypotensive response was significantly blunted (Fig. 3 and Table I).

Responses to the endogenous catecholamine, epinephrine (a combined  $\alpha$ -AR and  $\beta$ -AR agonist), were also significantly different between  $\beta 2$ -AR -/- and wild type mice (Fig. 4). In both  $\beta 2$ -AR -/- and wild type mice, administration of epinephrine produced a transient hypertensive response (blood pressure typically returned to baseline within 1 min). However, the hypertensive response was significantly greater in  $\beta 2$ -AR -/- mice than in wild types (Table I and Fig. 4). Heart rate responses in both  $\beta 2$ -AR -/- and wild type mice to epinephrine were variable (Table I). Although there was a trend for wild type mice to show heart rate increases while  $\beta 2$ -AR -/- showed heart rate decreases, these heart rate responses were not significantly different between genotypes.

The effects of exercise on heart rate and blood pressure are shown in Fig. 5. For these experiments, catheterized mice were tested using a graded exercise treadmill protocol.  $\beta 2$ -AR -/- and wild type mice showed similar heart rate increases during the exercise protocol. A significant difference, however, was observed in the blood pressure response to exercise. During the exercise protocol,  $\beta 2$ -AR -/- mice became hypertensive compared with wild type mice. At the peak exercise level of 20 m/min,  $\beta 2$ -AR -/- mice had a mean blood pressure of 139.3  $\pm$  4.4 mm Hg (mean  $\pm$  S.E.), whereas wild type mice had a mean blood pressure of 126.3  $\pm$  3.3 mm Hg (mean  $\pm$  S.E.).

**Metabolic Response to Exercise**—In a separate set of experiments, metabolic responses to exercise and exercise capacity were measured in uncatheterized mice. Oxygen consumption and carbon dioxide production were continuously monitored while the mice exercised according to a graded treadmill exercise protocol (Fig. 6). Oxygen consumption and carbon dioxide production were not significantly different between the two genotypes. However, there was a trend for  $\beta 2$ -AR -/- mice to

have greater levels of oxygen consumption at any given workload.  $\beta 2$ -AR -/- mice had a significantly lower respiratory exchange ratio during exercise than did wild type mice (Fig. 6C). There was also a significant difference between  $\beta 2$ -AR +/+ mice and  $\beta 2$ -AR -/- mice in exercise capacity. Interestingly,  $\beta 2$ -AR -/- mice exercised significantly longer than wild type control mice (Fig. 6D). Wild type mice covered 471  $\pm$  22 meters (mean  $\pm$  S.E.), whereas  $\beta 2$ -AR -/- mice covered 582  $\pm$  15 meters (mean  $\pm$  S.E.) during the graded exercise protocol.

**Body Weight, Body Fat, and Serum Free Fatty Acids**—To investigate possible mechanisms for the greater exercise capacity in  $\beta 2$ -AR -/- mice, we examined body weight, epididymal fat pad weight, body density, and serum levels of free fatty acid (FFA) and glycerol in wild type and  $\beta 2$ -AR -/- mice. As shown in Table II,  $\beta 2$ -AR -/- mice weigh significantly less than wild type mice. Epididymal fat pads from  $\beta 2$ -AR -/- mice also represent a smaller proportion of total body weight than fat pads from wild type mice. Previous studies have shown that the epididymal fat pad weight as a proportion of total body weight is highly correlated with total body fat in mice (20, 21). Body density, serum FFA levels, and serum glycerol levels were not significantly different between the two genotypes under baseline conditions.

**Locomotor Activity**—Activity studies were performed to determine if the observed differences in exercise capacity, body fat, and body weight in  $\beta 2$ -AR +/+ and  $\beta 2$ -AR -/- mice can be explained by differences in the level of daily activity. As shown in Fig. 7, there was no significant difference in locomotor activity between the two genotypes over a 48-h period. No significant differences were observed when the 48-h period was broken down into day and night segments (data not shown).

## DISCUSSION

Using gene targeting techniques we have generated mice that have a disruption in the  $\beta 2$ -AR gene. Based on ligand binding data this mutation blocks expression of the  $\beta 2$ -AR.  $\beta 2$ -ARs do not appear to play a critical role in prenatal development since there is no embryonic lethality associated with the mutation. Furthermore,  $\beta 2$ -AR -/- mice appear grossly normal and are fertile, demonstrating that  $\beta 2$ -ARs are not required for postnatal development or for normal reproductive function. There is no apparent compensatory up-regulation of  $\beta 1$ -ARs in the lungs of  $\beta 2$ -AR -/- mice.

**Cardiovascular Effects of the  $\beta 2$ -AR Gene Disruption**—One of the goals in generating  $\beta 2$ -AR -/- mice was to define further the roles of the three different  $\beta$ -AR subtypes in the cardiovascular system. A genetic approach has been used previously to show that  $\beta 1$ -AR and not  $\beta 2$ -AR stimulation regulates cardiac inotropy and chronotropy in the murine heart (10). Even though  $\beta 2$ -ARs are present in the myocardium of  $\beta 1$ -AR -/- mice, stimulation of  $\beta 2$ -ARs does not lead to improvements in

FIG. 3. *In vivo* cardiovascular responses to isoproterenol in  $\beta_2$ -AR  $+/+$  and  $-/-$  mice. After an overnight recovery from surgery, isoproterenol ( $3 \mu\text{g}/\text{kg}$ ) was administered intra-arterially. Representative tracings from a  $\beta_2$ -AR  $+/+$  and  $-/-$  mouse are shown. The upper trace represents heart rate, and the lower trace represents blood pressure. Isoproterenol was administered at the 1-min time mark.

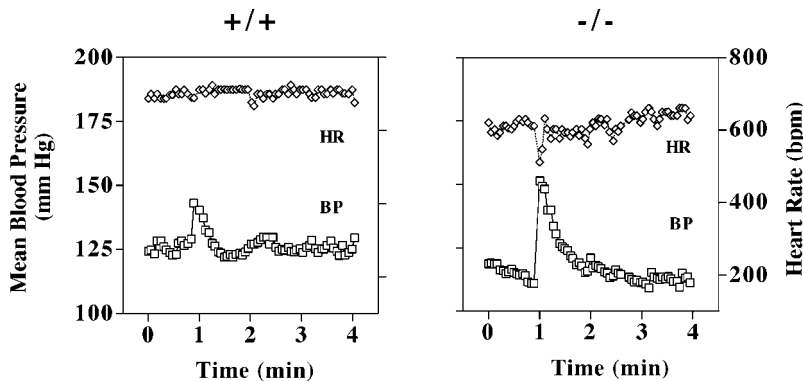
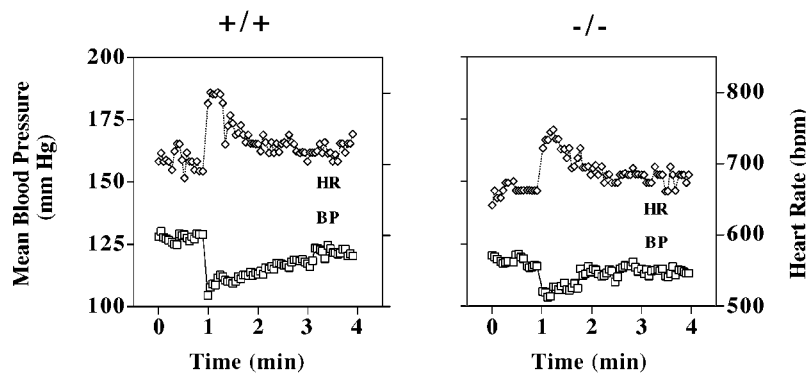
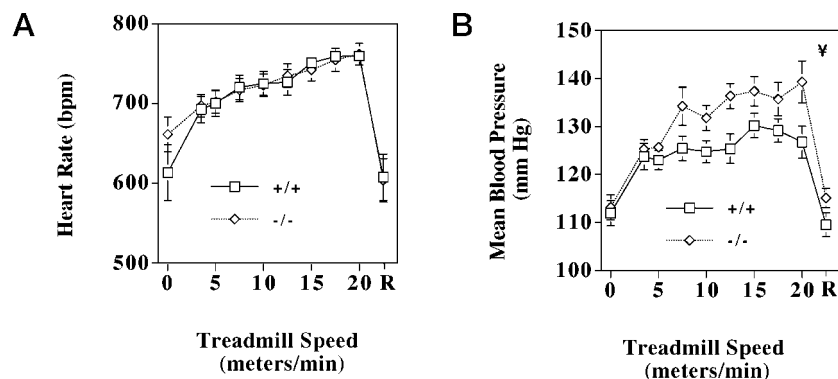


FIG. 4. *In vivo* cardiovascular responses to epinephrine in  $\beta_2$ -AR  $+/+$  and  $-/-$  mice. After an overnight recovery from surgery, epinephrine ( $3 \mu\text{g}/\text{kg}$ ) was administered through the arterial catheter. Representative tracings from a  $\beta_2$ -AR  $+/+$  and  $-/-$  mouse are shown. The upper trace represents heart rate, and the lower trace represents blood pressure. Epinephrine was administered at the 1-min time mark.

FIG. 5. Cardiovascular response to exercise in  $\beta_2$ -AR  $+/+$  and  $-/-$  mice. During a graded treadmill exercise program, heart rate (A) and blood pressure (B) were monitored in mice through the arterial catheter. Values shown represent the mean  $\pm$  S.E. for  $\beta_2$ -AR  $+/+$  ( $n = 8$ ) and  $\beta_2$ -AR  $-/-$  ( $n = 7$ ) mice.  $\text{¥}$ , significance at  $p < 0.001$  for comparing blood pressure from  $\beta_2$ -AR  $+/+$  mice to  $\beta_2$ -AR  $-/-$  mice by 2-way analysis of variance with repeated measures (treadmill speed  $\times$  genotype interaction); R, recovery phase defined as 10 min after stopping exercise.



cardiac function either *in vivo* or *in vitro*. In contrast, in  $\beta_2$ -AR  $-/-$  mice, normal heart rate responses are observed in response both to isoproterenol and to exercise, further demonstrating that the  $\beta_1$ -AR plays the major role in regulating cardiac function in the mouse.

Classical pharmacological studies have suggested that the  $\beta_2$ -AR is the  $\beta$ -AR subtype that mediates vascular smooth muscle relaxation (4, 5). Vascular relaxation leads to a decrease in total peripheral resistance and is manifested by a hypotensive blood pressure response. In  $\beta_2$ -AR  $-/-$  mice, the hypotensive response to isoproterenol is significantly blunted compared with wild type mice, confirming that  $\beta_2$ -ARs play a significant role in mediating peripheral vascular relaxation. The fact that hypotensive responses are still present in  $\beta_2$ -AR  $-/-$  mice, however, suggests that other  $\beta$ -AR subtypes also play a role, albeit a smaller one, in regulating peripheral vascular tone in the mouse. This response could also be due to an up-regulation of other  $\beta$ -AR subtypes in response to deletion of the  $\beta_2$ -AR gene; however, there was no evidence for compensatory up-regulation of the  $\beta_1$ -AR in the lung, the tissue with the highest density of  $\beta_2$ -ARs in wild type mice.

Although  $\beta_1$ -ARs are considered to be the cardiac  $\beta$ -AR, some previous studies support our conclusion that  $\beta_1$ -ARs can

mediate vascular relaxation.  $\beta_1$ -ARs have been shown to be involved in vascular relaxation in the isolated rat aorta and rat pulmonary artery (22).  $\beta_1$ -ARs have also been implicated in vascular relaxation in the coronary arteries from a variety of species (23–26). Although these larger vessels are not expected to make a significant contribution to the total peripheral resistance, studies on whole animals have also suggested that  $\beta_1$ -ARs may regulate resistance vessels in the peripheral vasculature (13).  $\beta_3$ -ARs, which are abundant in adipose tissue, have recently been shown to play a role in regulating peripheral vasodilation (11), although this was highly species-dependent (12). By generating double  $\beta_1$ -AR  $-/-$   $\beta_2$ -AR  $-/-$  mice, further insight into  $\beta_3$ -AR subtype function in the peripheral vasculature has been obtained (35).

*The Role of  $\beta_2$ -AR Receptors in Essential Hypertension*—In generating  $\beta_2$ -AR  $-/-$  mice, we were also interested in the possible role of  $\beta_2$ -ARs in hypertension (27). Hypertension is a complex disease with many environmental and genetic influences. Impaired  $\beta$ -AR-mediated vascular relaxation may contribute to this disease by preventing dynamic reductions in total peripheral vascular resistance. In animal models of hypertension,  $\beta$ -AR-mediated vascular relaxation has been shown to be attenuated (28). It is not clear from these studies, how-

**FIG. 6. Metabolic response to exercise and exercise capacity in  $\beta 2$ -AR  $+/+$  and  $-/-$  mice.** During a graded treadmill exercise protocol,  $O_2$  consumption (A),  $CO_2$  production (B), and the respiratory exchange ratio (C) were determined for each step in the exercise protocol. Values shown represent the mean  $\pm$  S.E. for  $\beta 2$ -AR  $+/+$  ( $n = 5$ ) and  $\beta 2$ -AR  $-/-$  ( $n = 5$ ) mice.  $O_2$  consumption and  $CO_2$  production are reported in units of ml/min/kg. The RER represents the ratio of  $CO_2$  production to  $O_2$  consumption. Exercise capacity (D) was measured as the total distance covered during the exercise protocol. Values shown represent the mean  $\pm$  S.E. for  $\beta 2$ -AR  $+/+$  ( $n = 5$ ) and  $\beta 2$ -AR  $-/-$  ( $n = 6$ ) mice.  $\text{¥}$ , significance at  $p = 0.0062$  for comparing exercise RER curves from  $\beta 2$ -AR  $+/+$  mice to  $\beta 2$ -AR  $-/-$  mice by 2-way analysis of variance with repeated measures (treadmill speed  $\times$  RER interaction); \*, significance at  $p = 0.0031$  for comparing  $\beta 2$ -AR  $+/+$  to  $-/-$  mice using an unpaired  $t$  test.

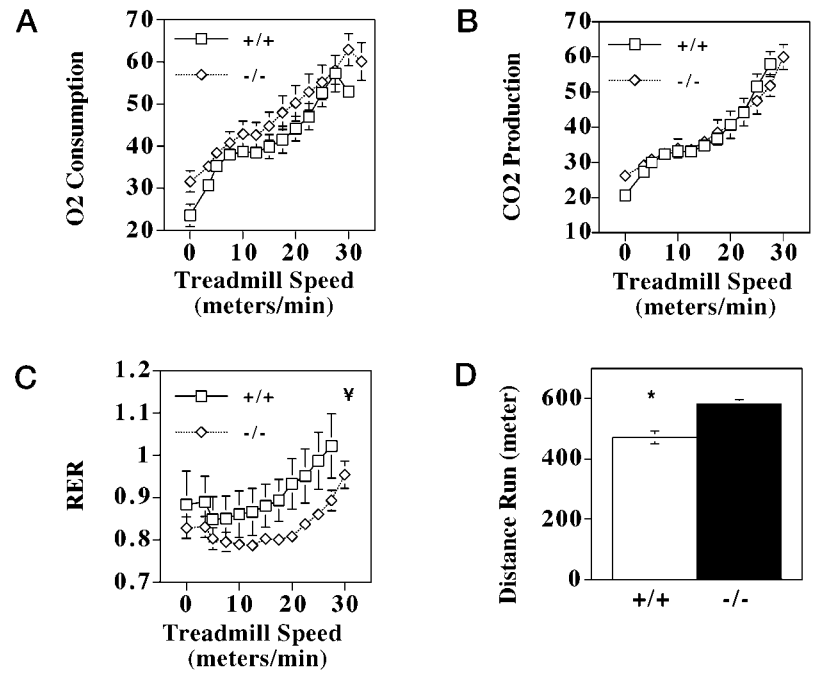


TABLE II

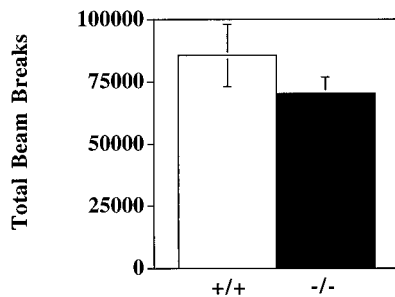
Body weight, proportional weight of the epididymal fat pads, density, free fatty acid (FFA) levels, and glycerol levels in  $\beta 2$ -AR  $+/+$  and  $\beta 2$ -AR  $-/-$  mice.

Values shown represent the mean  $\pm$  S.E. for  $\beta 2$ -AR  $+/+$  and  $\beta 2$ -AR  $-/-$  male mice. The unpaired  $t$  test was used for statistical comparison between groups. The numbers in parentheses represents the number of mice used for the study.

Genotype	Weight	Fat pad	Density	FFA	Glycerol
	g	% weight	g/ml	$\mu\text{mol/liter}$	mg/dl
$\beta 2$ -AR $+/+$	$32.31 \pm 0.65$ (11)	$3.01 \pm 0.27$ (11)	$0.942 \pm 0.006$ (11)	$2190 \pm 207$ (11)	$39.2 \pm 8.4$ (11)
$\beta 2$ -AR $-/-$	$29.12^a \pm 0.51$ (12)	$2.22^b \pm 0.12$ (12)	$0.943 \pm 0.005$ (12)	$2365 \pm 422$ (12)	$34.1 \pm 7.8$ (12)

<sup>a</sup> Significance at  $p = 0.0008$  for comparing  $\beta 2$ -AR  $+/+$  to  $-/-$  mice.

<sup>b</sup> Significance at  $p = 0.0118$  for comparing  $\beta 2$ -AR  $+/+$  and  $-/-$  mice.



**FIG. 7. Locomotor activity of  $\beta 2$ -AR  $+/+$  and  $-/-$  mice.** The total number of beam breaks for  $\beta 2$ -AR  $+/+$  ( $n = 5$ ) and  $\beta 2$ -AR  $-/-$  ( $n = 5$ ) mice is shown. Values shown represent the mean  $\pm$  S.E. The total number of beam breaks is not significantly different between the two genotypes ( $p > 0.05$  using an unpaired  $t$  test).

ever, whether decreased  $\beta$ -AR signaling is a primary cause of the hypertension or is a secondary effect, perhaps due to desensitization of  $\beta$ -ARs by chronically elevated catecholamines (29).  $\beta$ -AR-mediated vascular relaxation is impaired not only in animal models but also in humans with hypertension (30). Interestingly, humans with borderline hypertension have impaired  $\beta$ -AR-mediated vasodilation suggesting that defective  $\beta$ -AR signaling occurs early in the disease process and may be involved in its pathogenesis (30). A recent human population study has demonstrated that genetic variation of the  $\beta 2$ -AR is associated with a predisposition to develop hypertension (31). Defects in  $\beta 2$ -AR-mediated vasodilation may thus be a contributing factor to the development of hypertension.

In generating the  $\beta 2$ -AR  $-/-$  mouse we had the opportunity to test whether or not defective  $\beta 2$ -AR signaling can lead to a hypertensive state. Under baseline conditions we found that  $\beta 2$ -AR  $-/-$  mice are normotensive compared with wild type control mice. Thus,  $\beta 2$ -AR stimulation may not be involved in regulating resting blood pressure in mice. Given that  $\beta 2$ -AR  $-/-$  mice have had the disruption of the  $\beta 2$ -AR gene since conception, it is also possible that there have been other compensatory changes that allow the maintenance of resting blood pressure homeostasis. During the stress of treadmill exercise, however,  $\beta 2$ -AR  $-/-$  mice become hypertensive compared with wild type mice. As an endogenous stimulus for catecholamine release, exercise may lead to unopposed  $\alpha$ -adrenergic mediated vasoconstriction in the  $\beta 2$ -AR  $-/-$  mice. In support of this hypothesis, administration of epinephrine to the  $\beta 2$ -AR  $-/-$  mice reproduced the hypertensive phenotype. As a result of the disruption of the  $\beta 2$ -AR gene,  $\alpha$ -adrenergic receptor stimulation may predominate and predispose the animals to develop hypertension in states where endogenous catecholamines are elevated. In humans, hypertension worsens with the process of aging. In the present study, we studied young adult animals at 12–16 weeks of age. Future studies will be required to examine whether the  $\beta 2$ -AR  $-/-$  mice develop hypertension as they age.

**Metabolic Effects of the  $\beta 2$ -AR Receptor Gene Disruption—** $\beta 2$ -ARs are known to play a role in the metabolic response to stress (32). To investigate the effects of the knockout mouse on physiologic stress, metabolic responses to exercise and exercise capacity were measured in  $\beta 2$ -AR  $-/-$  and wild type mice.

Surprisingly,  $\beta$ 2-AR  $-/-$  mice exercised for a longer duration than wild type mice did. One explanation for this difference in exercise capacity is that there are alterations in energy metabolism secondary to ablation of the  $\beta$ 2-AR gene. In support of this hypothesis is the finding that  $\beta$ 2-AR  $-/-$  mice have a lower body fat content, and the respiratory exchange ratio (RER) was significantly lower in  $\beta$ 2-AR  $-/-$  mice at any given workload than in wild type mice during exercise. Although carbon dioxide production was similar between wild type and  $\beta$ 2-AR  $-/-$  mice, there was a trend toward higher oxygen consumption in the  $\beta$ 2-AR  $-/-$  mice. This trend for increased oxygen consumption during exercise may be explained by the lower body fat in  $\beta$ 2-AR  $-/-$  mice. Fat can be considered to be metabolically inert; therefore, changes in body fat content can also have an impact on oxygen consumption and carbon dioxide production when these parameters are normalized to total body weight. A change in body composition, however, does not explain the decrease in exercise RER in the  $\beta$ 2-AR  $-/-$  mice. RER is a ratio of oxygen consumption to carbon dioxide production; therefore, body composition factors are cancelled out by taking a ratio.

RER is an indicator of metabolic state and substrate utilization. Since more oxygen is required to burn fat than carbohydrate, a lower RER in  $\beta$ 2-AR  $-/-$  mice suggests that they may use a greater ratio of fat to carbohydrate than do wild type mice during exercise. This could also explain the lower body fat content in  $\beta$ 2-AR  $-/-$  mice. Previous studies (33, 34) have demonstrated that  $\beta$ -AR stimulation leads to glycogenolysis during exercise. If the knockout mice were to result in a defect in  $\beta$ 2-AR-mediated mobilization of glycogen,  $\beta$ 2-AR  $-/-$  mice may preferentially metabolize fat during exercise. Reduced utilization of the glycogenolysis pathway during exercise would conserve muscle glycogen and may be responsible for increasing the duration of exercise before glycogen depletion. We did not observe significant differences in serum-free fatty acids and glycerol in  $\beta$ 2-AR  $-/-$  mice under basal conditions; however, it is possible that differences in fat metabolism occur during exercise. Lactate production may also be influenced by the  $\beta$ 2-AR gene disruption. It has been shown that catecholamines stimulate lactate production, possibly through  $\beta$ 2-AR receptors (33, 34). Thus,  $\beta$ 2-AR  $-/-$  mice may have lower lactate levels for a given workload.

Other mechanisms may contribute to the increase in exercise capacity of  $\beta$ 2-AR  $-/-$  mice. There may be differences in the redistribution of cardiac output between visceral and peripheral muscular beds during exercise because of alterations in  $\beta$ 2-AR-mediated vasorelaxation. In the  $\beta$ 2-AR  $-/-$  mouse, the defect in  $\beta$ -AR-mediated vasorelaxation may attenuate the increase in flow to non-exercising tissues thereby allowing a larger percentage of the cardiac output to be diverted to skeletal muscle. The increased exercise capacity and reduced body fat content of  $\beta$ 2-AR  $-/-$  mice could reflect an elevated basal level of activity in these mice. However,  $\beta$ 2-AR  $+/+$  and  $\beta$ 2-AR  $-/-$  mice displayed similar 48-h activity levels (Fig. 7).

In summary, we have generated mice that have a targeted

disruption in the  $\beta$ 2-AR gene. These mice have normal resting heart rate and blood pressure but manifest hypertension in response to epinephrine infusion or to the cardiovascular stress induced by exercise.  $\beta$ 2-AR knockout mice should prove to be a useful model for further defining the roles of  $\beta$ -AR subtypes in cardiovascular, respiratory, metabolic, central nervous system, and reproductive functions.

*Acknowledgments*—We thank A. Nagy for the R1 embryonic stem cell line and J. M. Allen for the gift of the mouse  $\beta$ 2-AR genomic DNA clone.

#### REFERENCES

- Dixon, R. A., Kobilka, B. K., Strader, D. J., Benovic, J. L., Dohlman, H. G., Frielle, T., Bolanowski, M. A., Bennett, C. D., Rands, E., Diehl, R. E., Mumford, R. A., Slater, E. E., Sigal, I. S., Caron, M. G., Lefkowitz, R. J., and Strader, C. D. (1986) *Nature* **321**, 75–79
- Emorine, L. J., Marullo, S., Briand, S. M., Patey, G., Tate, K., Delavier, K. C., and Strosberg, A. D. (1989) *Science* **245**, 1118–1121
- Frielle, T., Collins, S., Daniel, K. W., Caron, M. G., Lefkowitz, R. J., and Kobilka, B. K. (1987) *Proc. Natl. Acad. Sci. U. S. A.* **84**, 7920–7924
- Lands, A. M., Arnold, A., McAuliff, J. P., Luduena, F. P., and Brown, T. G., Jr. (1967) *Nature* **214**, 597–598
- Lands, A. M., Luduena, F. P., and Buzzo, H. J. (1967) *Life Sci.* **6**, 2241–2249
- Strosberg, A. D. (1997) *Annu. Rev. Pharmacol. Toxicol.* **37**, 421–450
- Brodde, O. E. (1991) *Pharmacol. Rev.* **43**, 203–242
- Takei, M., Furukawa, Y., Narita, M., Murakami, M., Ren, L. M., Karasawa, Y., and Chiba, S. (1992) *Jpn. J. Pharmacol.* **59**, 23–30
- Kaumann, A. J. (1986) *Naunyn-Schmiedeberg's Arch. Pharmacol.* **332**, 406–409
- Rohrer, D. K., Desai, K. H., Jasper, J. R., Stevens, M. E., Regula, D. P., Jr., Barsh, G. S., Bernstein, D., and Kobilka, B. K. (1996) *Proc. Natl. Acad. Sci. U. S. A.* **93**, 7375–7380
- Shen, Y. T., Zhang, H., and Vatner, S. F. (1994) *J. Pharmacol. Exp. Ther.* **268**, 466–473
- Shen, Y. T., Cervoni, P., Claus, T., and Vatner, S. F. (1996) *J. Pharmacol. Exp. Ther.* **278**, 1435–1443
- Vatner, S. F., Knight, D. R., and Hintze, T. H. (1985) *Am. J. Physiol.* **H49**–H56
- Allen, J. M., Baetge, E. E., Abrass, I. B., and Palmiter, R. D. (1988) *EMBO J.* **7**, 133–138
- Soriano, P., Montgomery, C., Geske, R., and Bradley, A. (1991) *Cell* **64**, 693–702
- Nagy, A., Rossant, J., Nagy, R., Abramow-Newerly, W., and Roder, J. C. (1993) *Proc. Natl. Acad. Sci. U. S. A.* **90**, 8424–8428
- Joyner, A. L. (ed) (1993) *Gene Targeting: A Practical Approach*, pp. 33–61, Oxford University Press, Oxford
- Wood, S. A., Allen, N. D., Rossant, J., Auerbach, A., and Nagy, A. (1993) *Nature* **365**, 87–89
- Desai, K. H., Sato, R., Schauble, E., Barsh, G. S., Kobilka, B. K., and Bernstein, D. (1997) *Am. J. Physiol.* **H1053**–H1061
- Rogers, P., and Webb, G. P. (1980) *Br. J. Nutr.* **43**, 83–86
- Eisen, E. J., and Leatherwood, J. M. (1981) *Growth* **45**, 100–107
- O'Donnell, S. R., and Wanstall, J. C. (1984) *J. Pharmacol. Exp. Ther.* **228**, 733–738
- Hatakeyama, D. J., Stupecky, G. L., Purdy, R. E., and Choi, J. (1988) *Proc. West Pharmacol. Soc.* **31**, 171–172
- Nyborg, N. C., and Mikkelsen, E. O. (1985) *J. Cardiovasc. Pharmacol.* **7**, 1113–1117
- O'Donnell, S. R., and Wanstall, J. C. (1984) *Br. J. Pharmacol.* **81**, 637–644
- Toda, N., and Okamura, T. (1990) *J. Pharmacol. Exp. Ther.* **253**, 518–524
- Brodde, O. E., and Michel, M. C. (1992) *J. Hypertens. (suppl.)* **10**, S133–S145
- Feldman, R. D. (1987) *Can. J. Physiol. Pharmacol.* **65**, 1666–1672
- Goldstein, D. S. (1983) *Hypertension* **5**, 86–99
- Stein, C. M., Nelson, R., Deegan, R., He, H., Wood, M., and Wood, A. J. (1995) *J. Clin. Invest.* **96**, 579–585
- Timmermann, B., Mo, R., Luft, F. C., Gerdt, E., Busjahn, A., Omvik, P., Li, G. H., Schuster, H., Wienker, T. F., Hoehe, M. R., and Lund, J. P. (1998) *Kidney Int.* **53**, 1455–1460
- Opie, L. H. (1985) *Am. J. Cardiol.* **55**, 95D–100D
- Ahlborg, G. (1985) *Am. J. Physiol.* **E540**–E545
- Issekutz, B. J. (1984) *J. Appl. Physiol.* **57**, 1754–1759
- Rohrer, D. K., Chruscinski, A., Schauble, E. H., Bernstein, D., and Kobilka, B. K. (1999) *J. Biol. Chem.* **274**, 16701–16708