

Cardiovascular and Metabolic Alterations in Mice Lacking Both β 1- and β 2-Adrenergic Receptors*

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The activation state of β -adrenergic receptors (β -ARs) *in vivo* is an important determinant of hemodynamic status, cardiac performance, and metabolic rate. In order to achieve homeostasis *in vivo*, the cellular signals generated by β -AR activation are integrated with signals from a number of other distinct receptors and signaling pathways. We have utilized genetic knockout models to test directly the role of β 1- and/or β 2-AR expression on these homeostatic control mechanisms. Despite total absence of β 1- and β 2-ARs, the predominant cardiovascular β -adrenergic subtypes, basal heart rate, blood pressure, and metabolic rate do not differ from wild type controls. However, stimulation of β -AR function by β -AR agonists or exercise reveals significant impairments in chronotropic range, vascular reactivity, and metabolic rate. Surprisingly, the blunted chronotropic and metabolic response to exercise seen in β 1/ β 2-AR double knockouts fails to impact maximal exercise capacity. Integrating the results from single β 1- and β 2-AR knockouts as well as the β 1-/ β 2-AR double knockout suggest that in the mouse, β -AR stimulation of cardiac inotropy and chronotropy is mediated almost exclusively by the β 1-AR, whereas vascular relaxation and metabolic rate are controlled by all three β -ARs (β 1-, β 2-, and β 3-AR). Compensatory alterations in cardiac muscarinic receptor density and vascular β 3-AR responsiveness are also observed in β 1-/ β 2-AR double knockouts. In addition to its ability to define β -AR subtype-specific functions, this genetic approach is also useful in identifying adaptive alterations that serve to maintain critical physiological setpoints such as heart rate, blood pressure, and metabolic rate when cellular signaling mechanisms are perturbed.

The β -adrenergic receptors (β 1-, β 2-, and β 3-AR)¹ belong to the superfamily of G-protein-coupled receptors (1). Both sequence comparisons and functional studies suggest that these three receptors share many structural and mechanistic features (2). Agonist stimulation of cloned and exogenously expressed β -ARs has demonstrated that all three subtypes can couple through G_{α_s} to stimulate adenylate cyclase activity (3–

5). Despite these common structural and functional properties, however, individual β -AR subtypes *in vivo* remain as distinct therapeutic targets due to a number of factors that actually serve to distinguish them. These distinctions include tissue-specific expression patterns, the ability to couple to different G-proteins, pharmacological heterogeneity, and differences in agonist-dependent desensitization (6, 7).

β -AR subtypes can be distinguished pharmacologically by synthetic as well as natural ligands. The β 1-AR subtype shows little preference for epinephrine or norepinephrine, whereas the β 2-AR preferentially interacts with epinephrine (8, 9). More recent experiments demonstrate that the β 3-AR (previously termed “atypical”) preferentially interacts with norepinephrine over epinephrine. Synthetic subtype-selective agents have been developed which display much greater selectivity than these endogenous catecholamines. Some typical examples of these would include the antagonists CGP20712A (β 1-AR-selective) and ICI118551 (β 2-AR-selective) and the agonist CL316243 (β 3-AR-selective). Such synthetic compounds have proven invaluable for studying β -AR pharmacology and function (2, 10).

In vivo, β -ARs are known to modulate a wide range of physiological processes, from cardiac chronotropy and inotropy to vascular and smooth muscle tone, metabolism, and behavior. Functional assignment of β -AR subtype functions using pharmacological tools suggests that the β 1-AR is the predominant subtype regulating heart rate and contractility, although at least in the human, β 2-ARs are also thought to participate. β 2-ARs have been thought to be the predominant subtype mediating the vascular smooth muscle relaxant properties of β -AR agonists. The β 3-AR was initially identified and proposed to be the major β -AR subtype controlling lipolysis in adipose tissue. Although these functional divisions are not absolute, they appear to be well conserved across species and serve as a convenient framework for β -AR classification. However, defining β -AR subtype-specific functions *in vivo* can present significant challenges. Some subtype-selective agents display non-ideal behavior *in vivo*, either due to poor biodistribution or cross-reactivity with unrelated receptors. Gene disruption, or “knockout” experiments, has proven to be a useful approach in defining adrenergic receptor function *in vivo*. To date, this technique has been used to disrupt expression of all three α 2-AR subtypes, the α 1b-AR, the β 1-, and the β 3-ARs (11–16), and most recently, the β 2-AR (17). When the pharmacologic tools outlined above are used in conjunction with genetic techniques, the power to reveal novel functions and mechanisms of action can be greatly enhanced.

Given the prominent role of β -AR signaling in the maintenance of normal physiology *in vivo*, we sought to test the functional consequences of β -AR gene disruption via a combi-

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¹ The abbreviations used are: β -Ar(s), β -adrenergic receptor(s); GTE, graded treadmill exercise; Iso, isoproterenol.

natorial approach. In the companion article (17), the functional consequences of β 2-AR disruption are described. We have previously described the functional consequences of β 1-AR gene disruption (13, 18). We have now produced mice that lack both β 1- and β 2-ARs. The role of these two β -AR subtypes and the inferred role of the remaining β 3-AR subtype in cardiovascular physiology and metabolism are reported here.

MATERIALS AND METHODS

Generation of β -AR Knockout Mice—The generation of β 1-AR knockout mice has been previously described (13). Briefly, disruption of the β 1-AR gene was achieved using a positive-negative selection strategy to effect homologous recombination in the R1 embryonic stem cell line, using a targeting construct in which over 90% of the coding sequence was deleted. The strain background of β 1-AR knockout mice was a mixture of 129SvJ, C57Bl6/J, and DBA/2 which is less prone to the prenatal mortality previously described (13). The targeting strategy used to create β 2-AR knockout mice is described in the companion article (17) and is based on a similar positive-negative selection scheme and homologous recombination in the R1 embryonic stem cell line. Combination β 1/ β 2-AR double knockouts were generated by mating β 2-AR homozygous knockouts (on a combined 129SvJ and FVB/N mouse strain background) to homozygous β 1-AR knockouts. The resulting F1 generation of compound heterozygotes was subsequently intercrossed to generate F2 mice with all possible combinations of β 1- and β 2-AR gene disruptions. According to Mendelian inheritance, 1/16 of progeny were predicted to be homozygous-deficient for β 1- and β 2-AR, and 1/16 of progeny were predicted to be wild type for both β 1- and β 2-AR (see Table I). The F2 β 1/ β 2-AR double knockouts were bred to produce to double knockouts used in our experiments. The wild type F2 mice were bred to produce wild type controls. Thus, the overall strain contributions between wild type and β 1/ β 2-AR double knockouts were equivalent. Mice were genotyped for both β 1- and β 2-AR disruptions by Southern blotting of mouse tail biopsies (13, 17).

Mouse Instrumentation—Catheters were surgically implanted in either the left carotid artery or the left carotid artery plus the left jugular vein under isoflurane anesthesia. Briefly, anesthesia was induced with 3% (v/v) isoflurane in oxygen using an isoflurane vaporizer (Aircro Inc., Madison, WI), and then induction was maintained at 1.25–1.75% while monitoring the responsiveness of the animal. The vessels were cannulated with a stretched Intramedic PE10 polyethylene catheter (Clay Adams, Parsippany, NJ), which was filled with heparinized normal saline, sutured in place, and tunneled to the back. Blood pressure was measured using a DTX Plus pressure transducer (Spectramed, Oxnard, CA) amplified with a Gould 8-channel recorder, and the analog pressure was digitized using a Data Translation Series DT2801 analog-digital converter (Marlboro, MA). Digital signals were analyzed and stored using Crystal Biotech Dataflow data acquisition software (Crystal Biotech, Hopkinton, MA). Heart rate measurements were determined on-line and were derived from the pressure recordings. Drugs were infused through the arterial catheter as a bolus in a volume of 1–3 μ l/g. (–)-Isoproterenol hydrochloride (3 μ g/kg), atropine sulfate (1 mg/kg), epinephrine bitartrate (3 μ g/kg), and sodium nitroprusside (30 μ g/kg) were purchased from Sigma. CL316243 (100 μ g/kg) was a kind gift of Wyeth Ayerst Laboratories (Philadelphia, PA).

Assessment of Ventricular Function—A 1.4 French micromanometer-tipped Millar pressure transducer (Millar Instruments, Houston, TX) was advanced into the left ventricle via the right carotid artery under isoflurane anesthesia (see above). Correct placement of the catheter in the ventricle was judged by loss of the arterial waveform and transition to a waveform with similar peak systolic pressure, but diastolic pressures with minima in the 0–5 mm Hg range. Following correct placement, a jugular venous catheter was placed via the left jugular vein and advanced ~1 cm. The surgical incision was then sutured closed with 4-0 silk, and the mouse was allowed to stabilize for 10–15 min at 2.5% isoflurane. Pressure recordings were measured using a MacLab 8S digitizer/amplifier (MacLab, Milford, MA), recorded, and analyzed using MacLab/s version 3.5 software on a Macintosh 3400c. “Anesthetized” recordings of ventricular function were taken during a 1-min interval at the end of this 10–15-min equilibration period, at 2.5% isoflurane. Isoflurane anesthesia was then reduced in a stepwise fashion, from 2.5 to 1.25%, and mice were allowed to stabilize for 10 min. Following this period, isoflurane anesthetic was turned off, and the mouse was removed from the anesthetic nose cone and placed on its back. Upon self-righting (or the “awakening” state), mice were quickly euthanized with an intravenous dose of avertin. Awakening recordings of ventricular function were taken in the 30–60 s prior to the righting

response.

Exercise Protocols, Metabolic Measurements—Mice were subjected to either constant or graded treadmill exercise, using a Columbus Instruments Simplex II metabolic rodent treadmill, fitted with Oxymax oxygen and carbon dioxide gas analyzers (Columbus Instruments, Columbus, OH). For graded exercise, mice were placed in the exercise chamber and allowed to equilibrate (usually 30–60 min). 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. Pre-operatively, mice were initially subjected to this protocol, with regular stepwise increases until mice stopped running from exhaustion. Post-operatively, mice were run to a final end point of 20 m/min and 14° inclination. We have previously shown linear relationships between heart rate, VO_2 and VCO_2 during graded treadmill exercise in mice (19).

In Vitro Cardiac Physiology—The right ventricular free wall was dissected away from the left ventricle and interventricular septum, and silk sutures were tied at both ends of the long axis. Ventricles were placed in an oxygenated 32 °C tissue bath containing modified Krebs solution (118 mM NaCl, 5.4 mM KCl, 2.5 mM CaCl_2 , 0.57 mM MgSO_4 , 1.0 mM Na_2HPO_4 , 2.5 mM NaHCO_3 , 11.1 mM D-glucose). Ventricles were paced at 3.3 Hz by use of a Grass stimulator (30-ms pulse duration, 8–15 V). Signals from isometric force transducers were amplified and digitized with a MacLab 8S series amplifier and fed to MacLab/s version 3.5 software running on a Macintosh 3400c to determine twitch amplitude.

For spontaneously beating atria, right and left atria were dissected free of ventricular tissue, and both atrial appendages were tied with 4-0 silk sutures. These were placed in an oxygenated 32 °C tissue bath, where isometric force transduction and rate were monitored as above.

Radioligand Binding Assays—Ventricular homogenates were prepared by Polytron homogenization of whole organs in 5 mM Tris-Cl, 5 mM EDTA, pH 7.4, followed by centrifugation at 10,000 \times g. The resultant pellet was resuspended in 1 \times binding buffer (for α -adrenergic receptor binding, 150 mM NaCl, 50 mM Tris-Cl, 5 mM EDTA, pH 7.4; for muscarinic receptor binding, 75 mM Tris-Cl, 12.5 mM MgCl_2 , 1 mM EDTA, pH 7.4), and protein concentration was determined. For saturation binding, 50–100 μ g of homogenate protein was used in a 500- μ l reaction containing 300 pM ^{125}I -2-[β -(4-hydroxyphenyl)-ethyl-amino-methyl]tetralone or 300 pM [^3H]N-methyl scopolamine (both from NEN Life Science Products). Nonspecific binding was performed in duplicate with 20 μ M prazosin (Research Biochemicals, Natick, MA) or 5 μ M atropine sulfate, respectively (Sigma). All binding reactions were carried out at room temperature for ~2 h prior to vacuum filtration onto Whatman GF-C filters and determination of membrane-bound radioligand.

RESULTS

Generation and Recovery of β -AR Knockout Mice—The generation and viability of β 1-AR knockout mice (β 1-AR $-/-$) have been described previously (13). Briefly, homozygous β 1-AR knockouts derived from heterozygote:heterozygote matings (β 1-AR $+/-$ \times β 1-AR $+/-$) are recovered at an unexpectedly low frequency as predicted from Mendelian inheritance, although this effect can be ameliorated if the β 1-AR gene disruption is bred onto a multiple strain background. As described by Chruscinski *et al.* (17), the recovery of homozygous β 2-AR knockouts (β 2-AR $-/-$) is in accord with expected Mendelian frequencies.

Crosses were carried out between homozygous β 1-AR knockouts and homozygous β 2-AR knockouts to generate compound heterozygotes (β 1-AR $+/-$: β 2-AR $+/-$, see “Materials and Methods”), and these in turn were intercrossed to generate homozygous β 1- and β 2-AR double knockout mice (β 1-AR $-/-$: β 2-AR $-/-$). The expected frequency of recovering double knockout mice from compound heterozygote matings is 1 out of 16 or 6.25%. The observed frequency among weanlings was 7.23%, well within the expected range. Table I lists the expected and observed frequencies among the nine possible genotypes arising from the compound heterozygote intercrosses. The χ^2 distribution suggests that there are no significant deviations from Mendelian expectations either among individual genotypes or the group as a whole ($\chi^2 = 9.38$ with 8 degrees of

TABLE I
Frequency of viable pups at weaning from $\beta 1 +/ - ; \beta 2 +/ - \times \beta 1 +/ - ; \beta 2 +/ -$ intercrosses

The nine possible genotypes are listed together with the number of recovered viable pups. Expected values are derived from Mendelian inheritance patterns; $\chi^2 = \sum d^2/E$, where d is expected number - observed number, and E is expected number, with 8 degrees of freedom.

$\beta 1$ $\beta 2$	+/+ +/+	+/+ +/-	+/+ -/-	+/- +/+	+/- +/-	+/- -/-	-/- +/+	-/- +/-	-/- -/-	Totals
Observed	9	9	5	13	19	14	1	7	6	83
Expected	5.19	10.38	5.19	10.38	20.75	10.38	5.19	10.38	5.19	
χ^2	2.80	0.18	0.01	0.66	0.15	1.27	3.38	1.10	0.13	9.67

freedom, $p = 0.29$), although $\beta 1$ -AR knockouts ($\beta 1$ -AR $-/-$; $\beta 2$ -AR $+/+$) appear to be less well represented, in accord with our previous findings (13). Double knockout:double knockout matings were subsequently performed to generate mice for the studies reported here. Litter size, maternal behavior, and pup viability all appeared to be normal in this group.

Basal Cardiovascular Function—Basal cardiovascular parameters were measured in awake, unrestrained mice by use of indwelling carotid arterial catheters. As seen in Fig. 1, neither baseline heart rate (range 400–470 beats/min) nor mean arterial blood pressure (range 115–125 mm Hg) are significantly different when comparing wild type mice ($\beta 1$ -AR $+/+$; $\beta 2$ -AR $+/+$) to double knockouts ($\beta 1$ -AR $-/-$; $\beta 2$ -AR $-/-$).

Response to Catecholamines—Both isoproterenol and epinephrine were administered to wild type and $\beta 1/\beta 2$ -AR double knockout mice. The grouped response to these agents is shown in Fig. 2A. Whereas the non-selective β -AR agonist isoproterenol (3 $\mu\text{g}/\text{kg}$) elicits robust chronotropic and hypotensive responses in wild types, both of these responses are severely attenuated in $\beta 1/\beta 2$ -AR double knockout mice. Of note, both responses are also significantly time-delayed in $\beta 1/\beta 2$ -AR double knockouts in comparison to wild type responses. Furthermore, the small but significant increase in heart rate seen in double knockout mice in response to isoproterenol was attenuated by 93% in mice pretreated with the muscarinic antagonist atropine (1 mg/kg, data not shown), suggesting that the majority of this effect is due to the baroreflex, mediated by the vagus in response to the drop in blood pressure.

The effect of epinephrine (3 $\mu\text{g}/\text{kg}$) on $\beta 1/\beta 2$ -AR double knockouts is seen in the right-hand panel of Fig. 2A. This endogenous catecholamine is a mixed, non-selective α -AR and β -AR agonist. In wild types, this dose of epinephrine elicits tachycardia and a biphasic blood pressure response consisting of an initial brief hypertension followed by a more prolonged hypotensive response. In contrast, ablation of $\beta 1$ - and $\beta 2$ -AR signaling in the double knockout appears to convert this mixed α - and β -AR agonist into a selective α -AR agonist; these mice display concomitant bradycardia and a monophasic hypertensive blood pressure response. Again, the heart rate response to epinephrine seen in double knockouts appears to be predominantly due to baroreflex stimulation, as atropine pretreatment blocks 60% of this response (data not shown).

Fig. 2B is a compilation of the chronotropic and hemodynamic effects of isoproterenol on conscious and unrestrained $\beta 1$ -AR knockouts, $\beta 2$ -AR knockouts, and $\beta 1/\beta 2$ -AR double knockouts. These are all displayed relative to the response seen in wild type mice (dotted line at 100%) and represent the peak chronotropic and vasodilatory responses obtained in each genotype, respectively. Based on these data, ~50% of the chronotropic response to isoproterenol is lost when the $\beta 1$ -AR is knocked out, whereas there is no detrimental effect on heart rate in $\beta 2$ -AR knockouts. The combined $\beta 1$ - and $\beta 2$ -AR deficiency reduces the chronotropic response by over 85%. In terms of the vasodilatory response to isoproterenol, there appears to be a graded and additive attenuation of the hypotensive response with loss of the $\beta 1$ -AR (20% reduction), $\beta 2$ -AR (35% reduction), and combined $\beta 1/\beta 2$ -AR (71% loss).

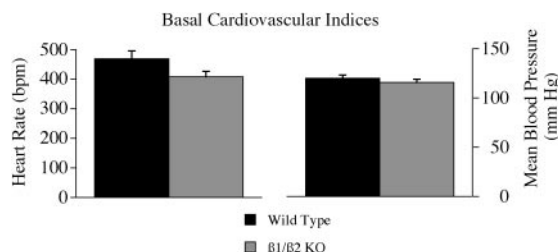


FIG. 1. Basal cardiovascular indices in wild type and $\beta 1/\beta 2$ knockout mice. Conscious, unrestrained mice instrumented with carotid arterial catheters were monitored for both heart rate (in beats per min (bpm)), and mean arterial blood pressure (mm Hg). 12 mice of each genotype were studied. $\beta 1/\beta 2$ KO refers to $\beta 1/\beta 2$ -AR double knockout mice.

Hemodynamic Responses to the $\beta 3$ -AR Agonist CL316243—The hemodynamic response to the $\beta 3$ -AR agonist CL316243 was tested in β -AR knockout mice to clarify the role of the $\beta 3$ -AR in the regulation of peripheral vasodilatory responses *in vivo*. Infusion of the $\beta 3$ -AR-selective agonist was followed by infusion of the non-selective β -AR agonist isoproterenol, to ascertain residual $\beta 1$ - and/or $\beta 2$ -AR responsiveness. Both drugs were used at doses that elicit maximal responses *in vivo* (19, 20). As can be seen in the top panel of Fig. 3, administration of CL316243 at 100 $\mu\text{g}/\text{kg}$ to a wild type mouse leads to a gradual but sustained hypotensive response. Near-maximal responses to this agonist were observed after 10 min, at which time isoproterenol was infused. These results clearly show that a residual $\beta 1$ - and/or $\beta 2$ -AR vasodilatory response can be elicited by isoproterenol even while $\beta 3$ -ARs are maximally stimulated. The time course of $\beta 3$ -AR-mediated vasodilatation suggests that the duration of action as well as the time interval to peak response is much longer for the $\beta 3$ -AR response to CL316243 than for the $\beta 1/\beta 2$ -AR response to isoproterenol. This appears to be unique to the response mediated by $\beta 3$ -ARs and not specific to CL316243, as isoproterenol given to $\beta 1/\beta 2$ -AR double knockouts also exhibits a similar time lag (Fig. 2A).

The bottom panel of Fig. 3 summarizes identically performed experiments on wild type, $\beta 1$ -, $\beta 2$ -, and combination $\beta 1/\beta 2$ -AR knockout mice. Interestingly, the response to the $\beta 3$ -AR agonist CL316243 alone was significantly augmented in the $\beta 1/\beta 2$ -AR double knockouts. Furthermore, the effect of isoproterenol following $\beta 3$ -AR stimulation (residual response) revealed that mice lacking $\beta 1$ -ARs showed no deficit in the residual vasodilatory response, whereas loss of $\beta 2$ -ARs had a large impact on further vasodilatory responses. Surprisingly, when both $\beta 1$ - and $\beta 2$ -ARs were lacking, isoproterenol infusion actually had a small hypertensive effect. Such a response can be due to either injection artifact or cross-reactivity to α -ARs. In either case, these results suggest that all three β -ARs can mediate vasodilatory responses *in vivo* and that an enhancement of $\beta 3$ -AR responsiveness is seen in mice lacking both $\beta 1$ - and $\beta 2$ -ARs.

Cardiovascular and Metabolic Responses to Exercise in the $\beta 1/\beta 2$ -AR Double Knockout—We also tested the role of $\beta 1$ -

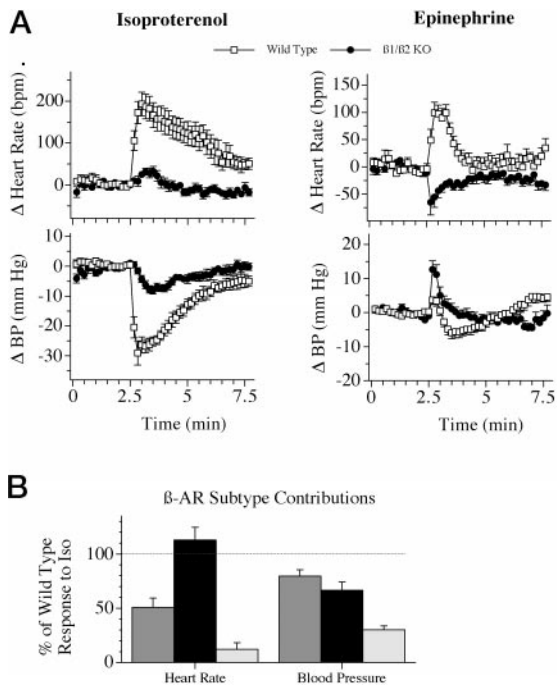


FIG. 2. Cardiovascular response to catecholamines. A, isoproterenol (3 μ g/kg) or epinephrine (3 μ g/kg) were injected intra-arterially as a bolus to conscious unrestrained wild type mice (squares) or β_1/β_2 knockouts (filled circles) at the 2.5-min time point. The effects on heart rate and blood pressure are shown for these two agents, expressed as the change (Δ) in beats/min (bpm) or mm Hg, respectively (wild type, $n = 10$; β_1/β_2 KO, $n = 9$). B, the percentage contribution of individual β -AR subtypes is inferred from a comparison of β_1 -, β_2 -, and β_1/β_2 -AR knockouts. As above, the response to isoproterenol (1–3 mg/kg intra-arterially) is shown as a percentage of the wild type response for either the increase in heart rate or the decrease in mean blood pressure. The dotted line indicates 100% of the wild type response (▨, β_1 knockouts, $n = 24$; ■, β_2 knockouts, $n = 16$; □, β_1/β_2 knockouts, $n = 9$). Data for β_1 knockouts were adapted from Rohrer *et al.* (18); data for β_2 knockouts were adapted from Chruscinski *et al.* (17). Responses in all groups except heart rate in β_2 knockouts are significantly decreased ($p \leq 0.01$) in comparison to the wild type response. For heart rate responses, all genotypes display significantly different heart rate responses from each other ($p \leq 0.02$). For blood pressure response, both β_1 and β_2 knockouts are significantly different from β_1/β_2 knockouts ($p \leq 0.01$).

and β_2 -AR signaling on the response to the physical stress of exercise. Knowing that β -ARs are recruited during exercise to modulate heart rate, hemodynamics, airway conductance, and metabolic rate, we hypothesized that mice lacking both β_1 - and β_2 -ARs would be compromised in both exercise capacity as well as the cardiovascular and metabolic response to exercise. Using graded treadmill exercise (GTE) as a stimulus, where both speed and angle of inclination are progressively increased, both wild type mice and β_1/β_2 -AR double knockouts were tested for total exercise capacity as well as the physiological response to fixed end point GTE. Total exercise capacity was measured as cumulative distance run in non-instrumented mice, with treadmill speed and angle of inclination increasing by 2.5 m/min and 2° every 3 min until mice stopped running from exhaustion. Physiological responses to fixed end point GTE were obtained by running instrumented mice to a final end point of 20 m/min and 14° inclination (see “Materials and Methods”).

Experiments designed to test total exercise capacity showed no significant differences between wild types and β_1/β_2 -AR double knockouts with respect to cumulative distance run. Wild type mice ran a total distance of 578.8 ± 33.3 m ($n = 7$), whereas β_1/β_2 -AR double knockouts ran a total distance of 545.2 ± 30.0 m ($n = 5$). The metabolic response to GTE in the maximal exercise capacity experiment is shown in Fig. 4B, demonstrating that whereas both wild types and β_1/β_2 -AR

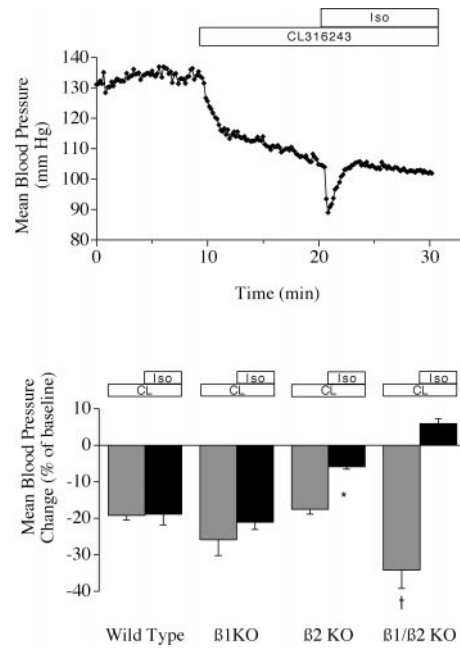


FIG. 3. Hemodynamic responsiveness to the β_3 -AR agonist CL316243 and the non-selective agonist isoproterenol. The upper panel is a representative blood pressure tracing of a wild type mouse given a single bolus injection of CL316243 (100 μ g/kg). Ten minutes following injection of the β_3 -AR agonist, isoproterenol was given (3 μ g/kg intra-arterially). The bar graph in the lower panel summarizes identically performed experiments for wild type, β_1 -, β_2 -, and β_1/β_2 -AR knockouts. The hemodynamic effect of CL316243 at 10 min (just prior to Iso administration) is shown by the cross-hatched bars, and the additional effect of Iso during the period just preceding Iso administration is shown by the black bars. Wild type, $n = 16$; β_1 knockouts (KO), $n = 6$; β_2 knockouts, $n = 9$; β_1/β_2 knockouts, $n = 7$. For β_2 knockouts, * indicates $p \leq 0.01$ versus wild type. For β_1/β_2 knockouts, † indicates $p \leq 0.01$ versus wild type.

double knockouts have virtually identical levels of O_2 consumption and CO_2 production at rest, consistent deficits in both of these indices are revealed at all exercise levels in the double knockout. This metabolic deficit appears to result from the combined deficiency of β_1 - and β_2 -ARs, since neither the β_1 -AR knockout nor the β_2 -AR knockout display such deficits (17, 18). Interestingly, however, there are differences in total exercise capacity between wild type mice and β_2 -AR knockouts, with the β_2 -AR knockout demonstrating a slight enhancement of total exercise capacity and reduced respiratory exchange ratios (17). In contrast, β_1 -AR ablation has no effect on total exercise capacity (18).

The physiological response to GTE is seen in Fig. 4A. Both blood pressure and heart rate were monitored in resting and exercising mice by use of indwelling carotid arterial catheters. The normal response to increasing exercise workloads is a corresponding increase in heart rate (up to the maximally achievable rate of ~ 800 beats/min in the mouse). Both wild type and β_1/β_2 -AR knockouts show workload-dependent increases in heart rate; however, the heart rate of β_1/β_2 -AR mice was lower than the heart rate of wild type mice at all exercise levels (at rest, heart rate differences are not statistically significant between the two genotypes). The effect of exercise on mean peripheral arterial blood pressure is not different between these two groups. The loss of exercise-induced tachycardia is most likely the result of β_1 -AR ablation, as a virtually identical behavior is seen in β_1 -AR knockout mice (18), whereas β_2 -AR knockouts show no deficit in exercise-induced tachycardia (17).

Muscarinic and α_1 -Adrenergic Receptor Density in Cardiac Membranes—There is a well known functional interdepend-

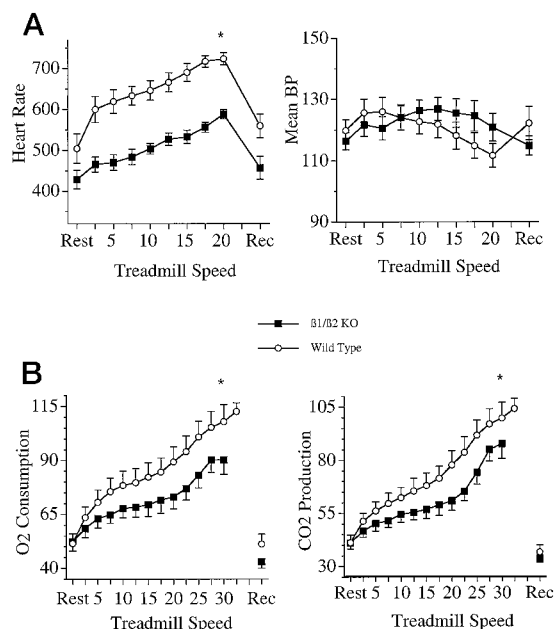


FIG. 4. Cardiovascular and metabolic response to exercise. Both wild type and β 1/ β 2-AR knockouts were subjected to a GTE regimen (see “Materials and Methods”). A, the cardiovascular response to GTE was determined in instrumented mice, which were run to a final end point of 20 m/min and 14° inclination. *Rec*, recovery (10 min post-exercise). Wild type, $n = 7$. β 1/ β 2 knockouts, $n = 6$. For heart rate, wild type *versus* β 1/ β 2 knockouts is significantly different ($p \leq 0.01$ by two-way analysis of variance with repeated measures, excluding recovery). B, the metabolic response to GTE was monitored in non-instrumented mice, which were run to their voluntary limit. O₂ consumption and CO₂ production are expressed as ml/min/kg. Wild type, $n = 7$. β 1/ β 2 knockouts, $n = 5$. Wild type mice are significantly different than β 1/ β 2 knockouts in both O₂ consumption and CO₂ production ($p \leq 0.01$ by two-way analysis of variance with repeated measures, up to 27.5 m/min treadmill speed).

ence between β -ARs and muscarinic receptors in the heart, which represent the two major targets of cardiac sympathetic and parasympathetic stimulation, respectively. Additionally, the role of α 1-ARs either alone or in combination with β -ARs is thought to be critical for both acute responsiveness to catecholamines, as well as in longer term adaptive or remodeling responses in the heart. We thus sought to test whether any gross alterations in either of these receptor families was apparent in β 1/ β 2-AR double knockouts in comparison to wild types. Whereas muscarinic receptor density displayed a mild but significant reduction in double knockouts in comparison to wild types (28.9 ± 1.6 fmol/mg protein *versus* 33.6 ± 1.0 fmol/mg protein; $n = 4$ for both, $p \leq 0.05$), α 1-AR density was not significantly affected by loss of both β 1- and β 2-ARs in comparison to wild types (50.3 ± 3.4 fmol/mg protein *versus* 54.1 ± 4.1 fmol/mg protein, respectively; $n = 4$ for both, $p =$ not significant).

In Vitro Cardiac Responsiveness to G-protein-coupled Receptor Agonists—The effect of various G-protein-coupled receptor agonists was tested in either spontaneously beating atrial preparations (chronotropic assay) or paced right ventricular strips (inotropic assay). These experiments were performed to investigate the efficacy of these compounds relative to β -AR agonists, as well as to reveal any potential compensation for loss of β -AR signaling that could be manifested as an altered response relative to wild type preparations. An additional utility of these experiments was the potential to reveal any compensation with an indirect mechanism of action, since several potential compensatory signaling pathways could exert their effects through modulating the release of catecholamines and subsequent activation of β -ARs (21–23). The agonists tested

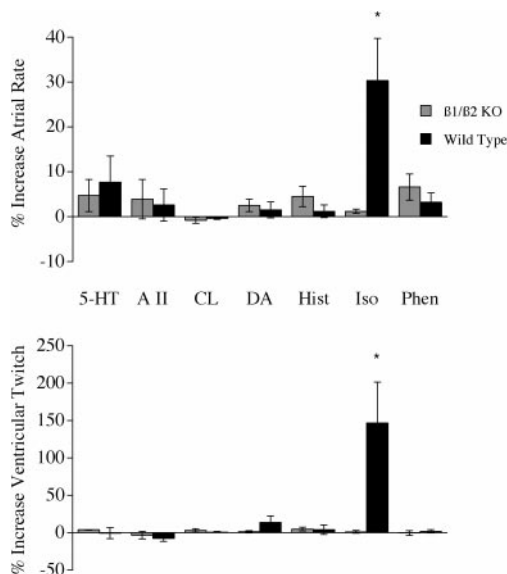


FIG. 5. In vitro chronotropic and inotropic responsiveness of wild type and β 1/ β 2-AR knockout preparations. A variety of pharmacological agents was tested for their ability to alter chronotropy or inotropy in spontaneously beating atrial preparations or paced right ventricular preparations, respectively. The upper panel shows the chronotropic response to serotonin (5-HT, 1 μ M), angiotensin II (A II, 0.1 μ M), the β 3-AR agonist CL316243 (CL, 1 μ M), dopamine (DA, 1 μ M), histamine (Hist, 1 μ M), isoproterenol (Iso, 1 μ M), and phenylephrine (Phen, 1 μ M), and the lower panel displays the inotropic response to the same agents. Wild type, $n = 3$. β 1/ β 2 knockouts, $n = 5$. All comparisons between wild type and β 1/ β 2 knockouts are not significant with the exception of Iso ($p \leq 0.05$ for both atrial rate and ventricular twitch response).

included serotonin, angiotensin II, the β 3-AR agonist CL316243, dopamine, histamine, the β -AR agonist isoproterenol (Iso), and the α -AR agonist phenylephrine. As can be seen in Fig. 5, there were no significant differences between wild type and β 1/ β 2-AR double knockout preparations in response to any of these drugs, with the exception of isoproterenol, which has robust effects on both atrial rate and ventricular contractility in wild type preparations but no effect in β 1/ β 2-AR double knockout preparations. The lack of effect of isoproterenol in the double knockout is virtually identical to that seen in β 1-AR knockout preparations (13), supporting the predominant role of β 1-ARs in the regulation of murine heart rate and contractility. The concentration of agonist used in each of these experiments (see Fig. 5 legend) was designed to elicit a maximal effect, based on prior experiments. It is notable that neither CL316243 nor isoproterenol has any appreciable effect on either atria or ventricles from β 1/ β 2-AR double knockouts, given the purported negative coupling behavior of the β 3-AR in human cardiac preparations (24).

In Vivo Left Ventricular Contractility in Anesthetized and Awakening Mice—Based on the observation that exercising β 1/ β 2-AR double knockout mice can achieve similar workloads at reduced heart rates, we sought to test whether corresponding deficits were also present in the inotropic component of heart function *in vivo*, given the well known effects of β -AR agonists to regulate cardiac contractility. These studies were performed in both anesthetized and awakening mice in an attempt to reduce the cardiodepressant effects of anesthesia, using micromanometer-tipped left ventricular catheters (see “Materials and Methods”). Fig. 6 shows representative tracings from two mice in both anesthetized and awakening states. Clearly both wild types and β 1/ β 2-AR double knockouts show depressed cardiac contractility, both in terms of developed pressure and dP/dt (the first derivative of left ventricular pres-

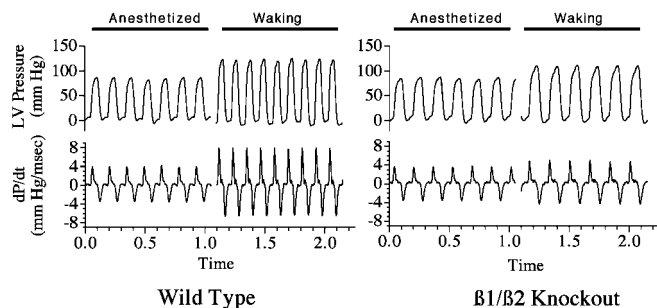


FIG. 6. Left ventricular contractility in anesthetized and awakening mice. Micromanometer-tipped pressure-sensing catheters were advanced from the carotid to the left ventricle under anesthesia, measurements were taken, and mice were allowed to recover until self-righting became evident. Representative tracings from two mice are shown, in both the anesthetized and awakening state. Both the left ventricular pressure tracings (*upper panels*) and the first derivative of pressure, dP/dt (*lower panels*), are shown for a wild type and $\beta 1/\beta 2$ -AR knockout mouse. Time axis is in seconds.

sure) in the anesthetized *versus* the awakening state. Furthermore, $\beta 1/\beta 2$ -AR double knockouts show diminished positive and negative peak dP/dt values relative to wild type mice whether measured during anesthesia or during awakening. Table II summarizes the data from these experiments, with the finding that while both $+dP/dt$ and $-dP/dt$ were reduced in $\beta 1/\beta 2$ -AR double knockouts relative to wild type mice (in both anesthetized and awakening states), there were no differences between wild types and double knockouts with respect to peak developed pressures in either state. There were also significant differences in heart rate between wild type and $\beta 1/\beta 2$ -AR double knockouts both in the anesthetized and awakening states, which in turn may influence dP/dt values. Monitoring of left ventricular pressure was terminated after mice righted themselves. At this time, the intraventricular pressure measurements became very unreliable and prone to position effects making these measurements in fully awake mice untenable.

DISCUSSION

The β -ARs are recognized as important components of the sympathetic nervous system, playing critical roles in the maintenance of cardiac, vascular, and metabolic homeostatic mechanisms. The purpose of these studies was to delineate the subtype-specific contributions of the $\beta 1$ -AR and $\beta 2$ -AR on these physiological processes by genetic knockout techniques, where $\beta 1$ - and $\beta 2$ -ARs were knocked out individually as well as in combination. By inference, we have also investigated cardiovascular functions specific to the remaining $\beta 3$ -AR. Surprisingly, total elimination of both $\beta 1$ - and $\beta 2$ -ARs has little impact on resting cardiovascular tone or basal metabolism, although functional deficits are clearly revealed when mice are stimulated by β -AR agonists or maximal exercise. Such results underscore the notion that the β -ARs are modulators of these physiologic functions but not intrinsic to or required for the functions themselves.

Basal Cardiovascular Function—Loss of both $\beta 1$ - and $\beta 2$ -ARs has minimal impact on basal heart rate and blood pressure. Based on the phenotype of both the $\beta 1$ -AR knockout (13, 18) and the $\beta 2$ -AR knockout (17), these results are not surprising. Such results would be unexpected, however, when considered in the context of numerous pharmacological studies using either non-selective or selective β -AR antagonists that are commonly used to lower heart rate and blood pressure. Why do mice lacking both adrenergic receptors fail to exhibit abnormalities at rest? First, there may be fundamental differences between animals that lack a given receptor from conception onwards and animals treated with antagonists at a discrete

point in time. Furthermore, the bulk of evidence from knockouts of the $\alpha 1b$ -, $\alpha 2a$ -, $\alpha 2b$ -, $\alpha 2c$ -, and $\beta 3$ -ARs also reveals that basal physiological functions are not significantly perturbed, again failing to reproduce the phenotypes observed by acute subtype-specific blockade in normal animals (25). Our data suggest that there are alternative control points for such critical physiological functions such as cardiac rate and contractility, vascular tone, and metabolic state, which can be altered to compensate for the lack of β -AR signaling. The parasympathetic nervous system, which acts in functional opposition to signals generated by the sympathetic nervous system (26, 27), has the potential to compensate for absent β -AR signaling, as do a variety of other hormone or neurotransmitter systems. Our demonstration that cardiac muscarinic receptor density is reduced in the $\beta 1/\beta 2$ -AR double knockout may reflect a counterbalancing reduction in a receptor that is known to functionally antagonize stimulatory β -ARs.

$\beta 3$ -AR Function—Another example of a compensatory change in G-protein-coupled receptor signaling resulting from $\beta 1$ - and $\beta 2$ -AR deficiency is the supranormal response of $\beta 1/\beta 2$ -AR knockouts to the $\beta 3$ -AR agonist CL316243. Stimulation of $\beta 3$ -ARs by this agonist (and 37344 from Life Technologies, Inc.) in rats and dogs elicits sustained decreases in both blood pressure and total peripheral resistance (20). In wild type mice, we have demonstrated that $\beta 3$ -AR stimulation also results in a robust and sustained hypotensive response. Mice deficient in both $\beta 1$ - and $\beta 2$ -ARs show an exaggerated response to CL316243. There are several possible explanations for such altered responses. First, vascular $\beta 3$ -ARs may be up-regulated in the $\beta 1/\beta 2$ -AR double knockout. The demonstration that $\beta 1$ -ARs are up-regulated in adipose tissue of $\beta 3$ -AR knockout mice (12) supports the contention that deficiencies in β -AR signaling can be counteracted by increases in the density and/or signaling efficiency of other β -AR subtypes. Another possibility is that there is an up-regulation of the signaling machinery distal to β -ARs in the vascular beds of $\beta 1/\beta 2$ -AR double knockouts, secondary to disuse. The phenomenon of β -AR supersensitization following prolonged β -AR antagonist therapy is well known (28–30) and may be analogous to the situation in mice when both $\beta 1$ - and $\beta 2$ -ARs are absent.

Experiments with the $\beta 3$ -AR agonist CL316243 demonstrate that residual responses (defined as the additional isoproterenol-induced vasodilatory response during full $\beta 3$ -AR stimulation) of $\beta 1$ -ARs differ from that of $\beta 2$ -ARs in mice which lack one or the other subtype. In these experiments, $\beta 1$ -AR knockouts possess identical residual isoproterenol responses in comparison to wild types, whereas $\beta 2$ -AR knockouts showed attenuated residual responses. This could be due to either increased efficacy of the $\beta 2$ -AR in mediating vasodilatation, different mechanism(s) of receptor activation, or to differences in the distribution among the three β -ARs within vascular beds. As an example, preferential distribution of $\beta 2$ -ARs in small resistance arterioles and $\beta 1$ -ARs in large conductance vessels would tend to favor $\beta 2$ -ARs in the primary control of peripheral vascular resistance. Additionally, the attenuated response of $\beta 2$ -AR knockouts to isoproterenol in these experiments could indicate an overlap of $\beta 1$ - and $\beta 3$ -AR expression in the same vascular beds, such that $\beta 3$ -AR stimulation with CL316243 precludes a maximal response through $\beta 1$ -ARs co-expressed in the same resistance vessels.

Metabolic and Physiologic Response to Exercise—The ability of β -AR antagonists to alter the metabolic response to exercise is well known (31–33). β -AR activation normally stimulates glycogenolysis as well as lipolysis, reflected in the rise of plasma glucose and free fatty acids during exercise. Whereas free fatty acid mobilization is impaired in β -AR-blocked exer-

TABLE II
Summary of left ventricular contractility measurements

Heart rate (HR), maximum left ventricular pressure (Max LVP), minimum left ventricular pressure (Min LVP), maximum dP/dt (Max dP/dt), and minimum dP/dt (Min dP/dt) are shown for both wild types and $\beta 1/\beta 2$ -AR knockouts under anesthetized and awakening conditions. Wild type, $n = 5$; $\beta 1/\beta 2$ knockouts, $n = 6$.

	Anesthetized					Waking				
	HR	Max LVP	Min LVP	Max dP/dt	Min dP/dt	HR	Max LVP	Min LVP	Max dP/dt	Min dP/dt
	<i>beats/min</i>	<i>mm Hg</i>		<i>mm Hg/ms</i>		<i>beats/min</i>	<i>mm Hg</i>		<i>mm Hg/ms</i>	
Wild type (5)	384.6 $\pm 22.9^a$	100.0 $\pm 3.6^a$	-0.3 $\pm 2.4^a$	5.00 $\pm 0.19^a$	-3.90 $\pm 0.36^a$	471.6 ± 27.0	122.2 ± 4.2	-8.9 ± 1.1	8.28 \pm 0.6	-6.46 ± 0.48
$\beta 1/\beta 2$ knockout (6)	316.0 $\pm 12.4^b$	94.8 $\pm 4.9^a$	2.4 ± 3.2	4.03 $\pm 0.29^{a,b}$	-3.4 $\pm 0.11^{a,b}$	331 $\pm 8.7^b$	116.9 ± 5.4	1.8 $\pm 3.3^b$	5.55 $\pm 0.72^b$	-4.05 $\pm 0.25^b$

^a $p < 0.05$, anesthetized versus waking, within a genotype.

^b $p < 0.05$ wild type versus $\beta 1/\beta 2$ knockout.

cising subjects, glycogen utilization is unimpaired, and there appears to be no change in the glucagon response (31–33). In addition to the increased mobilization of metabolic fuels, there are a variety of downstream effectors of β -AR stimulation that can contribute to increased metabolic demands as follows: adenylyate cyclase, the Na^+/K^+ -ATPase, and the L-type Ca^{2+} channel are all activated by β -AR stimulation (34–36). Thus, it is not surprising that metabolic demands are reduced in $\beta 1/\beta 2$ -AR knockout mice during exercise, although it is surprising that these mice show no impairment of total exercise capacity when run to exhaustion. It is interesting to note that the metabolic alteration seen in $\beta 1/\beta 2$ -AR double knockouts appears to result from combined $\beta 1$ - and $\beta 2$ -AR deficiency, as neither single $\beta 1$ - nor $\beta 2$ -AR knockouts display the same degree of metabolic hypo-responsiveness during exercise. In humans, such metabolic responses were traditionally thought to be regulated primarily by the $\beta 2$ -AR (31), although there are instances where both $\beta 1$ - and $\beta 2$ -ARs appear to be involved in the metabolic response to exercise (37, 38). Our results in mice suggest that both the $\beta 1$ - and $\beta 2$ -AR normally subserve redundant metabolic functions *in vivo* and that both receptor subtypes must be eliminated before significant defects are manifested.

The study of receptor pharmacology in conscious animals is frequently complicated by responses that are either indirect or reflexive in nature. In the present study, the chronotropic effect of isoproterenol in the $\beta 1/\beta 2$ -AR double knockout is almost completely eliminated by pretreatment with atropine and thus must be primarily a reflex response to $\beta 3$ -AR-mediated vasodilatation. In a previous study using atropine, we demonstrated that ~50% of the typical heart rate response to isoproterenol in conscious mice is due to parasympathetic withdrawal, and ~50% is due to direct $\beta 1$ -AR stimulation (18). A reflex mechanism for $\beta 3$ -AR stimulation of heart rate is supported by studies in dogs (20) and by our own studies on isolated atria, in which $\beta 1/\beta 2$ -AR knockout preparations fail to demonstrate any direct inotropic or chronotropic effects from either isoproterenol or the $\beta 3$ -AR agonist CL316243. In some instances, we were not able to block completely the baroreflex with atropine, as evidenced by the bradycardic effect of epinephrine in the $\beta 1/\beta 2$ -AR double knockouts. These results suggest that either atropine dosage was not sufficiently high to block the increase in vagal tone following a hypertensive stimulus or that an α -AR component of the action of epinephrine has negative chronotropic effects. The failure to demonstrate significant chronotropic or inotropic effects of agents such as histamine or serotonin, despite their ability to stimulate cardiac adenylyate cyclase *in vitro* (39, 40), suggests that β -ARs are the primary G-protein-coupled receptors regulating cardiac function *in vivo*.

Given that both $\beta 1$ -AR knockouts (18) and the $\beta 1/\beta 2$ -AR

double knockout show normal exercise capacities at submaximal heart rates, we wanted to investigate whether there were any significant differences in cardiac inotropic state that could represent an adaptation to the loss of β -ARs. Based on our previous findings with $\beta 1$ -AR knockouts (13, 41), the inotropic state is largely determined by the presence or absence of the $\beta 1$ -AR and any underlying sympathetic tone. Our results here suggest that there may be some residual sympathetic tone in anesthetized mice, which in wild type mice manifests itself as an increased $+dP/dt$ and decreased $-dP/dt$ in comparison to the $\beta 1/\beta 2$ -AR double knockout; this is even further accentuated in the awakening state. Alternatively, the difference in heart rates between wild types and double knockout mice during anesthesia and upon awakening could be responsible for this difference in dP/dt . Interestingly, inotropic state is greatly enhanced even in $\beta 1/\beta 2$ -AR double knockouts upon transition from anesthetized to awakening states. Depression of myocardial contractility while under isoflurane anesthesia can be due to indirect effects to reduce sympathetic outflow, as well as direct inhibitory effects on cardiac muscle (42–44). Despite the reduced positive and negative dP/dt values in $\beta 1/\beta 2$ -AR double knockouts, these mice develop equivalent peak left ventricular pressures in comparison to their wild type counterparts. Taken together with the exercise studies, our results would suggest that the β -AR-mediated increases in heart rate and contractility in the mouse are not necessary for maximal performance during a stress such as exercise. In fact, at the extremely high heart rates typical for a mouse at maximal exercise, reduced diastolic filling time may serve to limit any further increases in cardiac output. The demonstration that humans under β -AR blockade can increase stroke volume via the Frank-Starling mechanism (while heart rate remains depressed) supports the idea that chronotropic and inotropic stimulation through β -ARs are not required for maximal exercise performance (45). Other studies have shown that during exercise, intrinsic mechanisms such as increased venous return enhances diastolic filling and hence cardiac output (46, 47) and can be preferentially utilized over heart rate changes in some pathological states to maintain cardiac output (48). Together with our data in genetically altered mice, such results underscore the importance of intrinsic preload and afterload mechanisms and tend to mitigate the requirement for β -AR signaling in the adaptive responses to exercise.

In summary, the present study has further characterized the physiological role of $\beta 1$ - and $\beta 2$ -ARs in mice by means of genetic knockout techniques. The impact of $\beta 1$ -, $\beta 2$ -, or $\beta 1/\beta 2$ -AR loss on basal physiological functions such as heart rate, blood pressure, or metabolic rate is remarkably minor; however, striking differences between these knockouts and their wild type counterparts can be seen following β -agonist administration or during the stresses of exercise. Given what is known

about the important role that β 1- and β 2-ARs play in both physiological and pathophysiological processes, one can speculate as to whether the gene knockout technique has revealed the "true" role of these receptor subtypes. More thorough studies involving different types of stresses (such as induced heart failure) and longitudinal studies will help to clarify this issue. Despite the limitations of the model systems studied to date, certain β -AR-modulated functions such as heart rate and contractility are well defined and can be attributed to single β -AR members (in this case the β 1-AR). Other functions, such as β -AR-mediated vasodilatation, are additive and integrated, with all three β -AR subtypes contributing at some level. For other functions, such as metabolic rate, β -AR actions appear to be redundant, and deficiencies are not apparent until both β -AR subtypes are knocked out. Mice lacking β 1- and/or β 2-ARs represent useful model systems for the study of β -AR-modulated function *in vivo*, as well as the role that β -ARs play in pathophysiology.

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