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Article

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β_3 -adrenoceptor deficiency blocks nitric oxide-dependent inhibition of myocardial contractility

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The cardiac β -adrenergic pathway potently stimulates myocardial performance, thereby providing a mechanism for myocardial contractile reserve. β -Adrenergic activation also increases cardiac nitric oxide (NO) production, which attenuates positive inotropy, suggesting a possible negative feedback mechanism. Recently, in vitro studies suggest that stimulation of the β_3 -adrenoceptor results in a negative inotropic effect through NO signaling. In this study, using mice with homozygous β_3 -adrenoceptor deletion mutations, we tested the hypothesis that the β_3 -adrenoceptor is responsible for β -adrenergic activation of NO. Although resting indices of myocardial contraction were similar, β -adrenergic-stimulated inotropy was increased in $\beta_3^{-/-}$ mice, and similar hyper-responsiveness was seen in mice lacking endothelial NO synthase (NOS3). NOS inhibition augmented isoproterenol-stimulated inotropy in wild-type (WT), but not in $\beta_3^{-/-}$ mice. Moreover, isoproterenol increased myocardial cGMP in WT, but not $\beta_3^{-/-}$ mice. NOS3 protein abundance was not changed in $\beta_3^{-/-}$ mice, and cardiac β_3 -adrenoceptor mRNA was detected in both NOS3^{-/-} and WT mice. These findings indicate that the β_3 -adrenergic subtype participates in NO-mediated negative feedback over β -adrenergic stimulation.

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Introduction

Nitric oxide (NO) inhibition of β -adrenergic-stimulated contractility has been demonstrated in vitro (1) and in vivo (2–5). In addition to activating adenylyl cyclase production of cAMP, a cardiostimulatory pathway, β -adrenergic agonists lead to the production of NO. NO stimulates soluble guanylyl cyclase to produce cGMP, a cyclic nucleotide that opposes the cardiostimulatory effects of cAMP. Cardiodepression due to the NO-cGMP pathway likely has pathophysiologic significance because its activity is increased with heart failure (6, 7), sepsis (8), and aging (9, 10).

Whereas β -adrenergic stimulation has been shown to increase NO production (11), the relative contributions of specific β -adrenoceptor subtypes (1, 2, or 3) in this signal-transduction pathway have not been established. The β_3 -adrenoceptor, recently appreciated in the mammalian heart (12–14), is widely expressed in adipose, intestinal, and lung tissues (15), where its activation promotes lipolysis and energy expenditure (16–18). β_3 -adrenoceptor activation in the human heart produces direct negative inotropic responses (12, 13, 19). Observations that these negative inotropic effects are attenuated by NO synthase (NOS) inhibition suggest that the β_3 -adrenoceptor has a role in NO-mediated inhibition of adrenergic stimulation.

The lack of specific β_3 -adrenoceptor antagonists has hampered characterization of the precise in vivo role of β_3 -NO signaling. To address this issue we measured β -adrenergic contractility in mice with a homozygous deletion mutation of the β_3 -adrenoceptor gene ($\beta_3^{-/-}$). Using these animals, we tested the hypothesis that $\beta_3^{-/-}$ mice lack the NO negative feedback over β -adrenergic contractility.

Methods

Murine species. $\beta_3^{-/-}$ mice (34 ± 1 g body weight, 7–9 months old), based on an inbred FVB background, were obtained courtesy of Brad Lowell, Beth Israel-Deaconess Medical Center (Boston, Massachusetts, USA) (20–22). FVB wild-type (WT) mice (32 ± 1 g body weight, 7–9 months old) from Taconic Farms (Germantown, New York, USA) were used as control animals. NOS3^{-/-} mice (31 ± 2 g body weight, 7–9 months old) were kindly provided by Paul Huang (Massachusetts General Hospital, Boston, Massachusetts, USA) (23).

Animal preparation. Animals were housed under diurnal lighting conditions and allowed food and tap water ad libitum. Animal treatment and care was provided in accordance with institutional guidelines, and the protocol was approved by the Animal Care and Use Committee of the Johns Hopkins University.

Mice were anesthetized and ventilated as described previously (24). Briefly, mice were anesthetized with a combination of etomidate (3.3 mg/kg), urethane (1000 mg/kg), and morphine (0.5 mg/kg). A tracheotomy was performed, and mice were ventilated at 120 breaths per minute with a peak airway pressure of less than 11 mmHg. After a substernal lateral thoracotomy, a combined micromanometer-conductance catheter (SPR-719; Millar Instruments Inc., Houston, Texas, USA) was advanced retrograde into the left ventricle (LV) through an apical stab wound made with a 25-gauge needle, along the cardiac longitudinal axis; the distal tip was placed in the aortic root and a proximal electrode was placed just within the endocardial wall of the LV apex.

Infusions were administered through the right jugular vein cannulated with a 30-gauge needle. Offset calibration of the recorded volume signal was obtained by the saline wash-in technique (25, 26). Stroke volume calibration was derived from the cardiac output obtained from direct measurements of the aortic blood flow, obtained using a flow probe (AT01RB; Transonic Systems Inc., Ithaca, New York, USA) placed around the aorta, and the flow per minute was recorded (AT106; Transonic Systems Inc.). Pressure, volume, and flow signals were digitized at 1 kHz, stored to a disk, and analyzed with custom software.

Hemodynamic data analysis. Indices of myocardial systolic and diastolic performance were derived from pressure-volume data obtained both at steady state and during transient loading of the heart with direct occlusion of the abdominal aorta. Cardiac preload was indexed as the left ventricular end-diastolic volume (EDV) and end-diastolic pressure (EDP). Cardiac afterload was evaluated as effective arterial elastance (E_a ; ratio of LV systolic pressure to stroke volume) (27, 28). This parameter is not preload dependent and has been validated to closely approximate total afterload, which incorporates systemic vascular resistance, aortic impedance, and the reflected wave properties of the vasculature. Myocardial contractility was indexed by the peak rate of rise in LV pressure (+dP/dt) divided by instantaneous pressure (dP/dt-IP) (29) and the load-independent end-systolic elastance (E_{es}) (30, 31). E_{es} is the slope of the end-systolic pressure-volume relationship (ESPVR). Because the ESPVR is nonlinear in mice, we used the formula derived by Mirsky (32) to fit our pressure-volume data:

(Equation 1)

$$P_{es} = \frac{1}{\alpha + \beta \cdot V_{es}} \log \left[\frac{V_{es}}{V_0} \right]$$

Where α and β are fitting parameters, P_{es} and V_{es} are the end-systolic pressure and volume, respectively, and V_0 is the volume of the heart at zero pressure. This formula is derived from the mechanical properties of muscle and has been validated in the canine heart (32). E_{es} was taken as the average slope of the fitted ESPVR in the pressure range of 90–160 mmHg. Diastolic per-

formance was measured by peak – dP/dt, and the time constant of ventricular relaxation (τ) (33).

Experimental protocol and drugs. Human albumin (220 μ L of 8.3% in normal saline; Central Laboratory, Blood Transfusion Service, Swiss Red Cross, Bern, Switzerland) was given to maintain intravascular volume over 2 minutes, 5 minutes before the drug infusion. Concentration-effect curves to isoproterenol (0.2 mg/mL dissolved in normal saline) were generated in $\beta_3^{-/-}$ ($n = 7$) and WT ($n = 12$) mice. These mice were given isoproterenol at 1, 10, and 100 ng/kg/min for 4 minutes each at rates of 5–9 μ L/min. Control experiments showed that the vehicle itself, at the experimental infusion rates, has no effect on cardiovascular performance.

In additional mice, isoproterenol was infused intravenously at a rate of 5 ng/kg/min, at 5–9 μ L/min to WT ($n = 8$), $\beta_3^{-/-}$ ($n = 10$), and $NOS3^{-/-}$ ($n = 15$) mice for 4 minutes. In these WT and $\beta_3^{-/-}$ mice, L- N^G -monomethyl arginine (L-NMMA; Calbiochem-Novabiochem, La Jolla, California, USA) was dissolved in normal saline and administered as a co-infusion with the isoproterenol at the rate of 10 mg/kg/h for 5 minutes. Another NOS inhibitor, L- N^G -nitroarginine methyl ester hydrochloride (L-NAME, 0.1 mg/kg/h) was also co-infused with isoproterenol in WT ($n = 3$) and $\beta_3^{-/-}$ ($n = 4$) mice.

NOS protein abundance. Western blot analysis was performed on total protein isolated from mouse hearts and separated on 7.5% Tris-Glycine Ready Gel (Bio-Rad Laboratories, Richmond, California, USA). The protein was then transferred to a nitrocellulose membrane and nonspecific binding was blocked overnight at 4°C in 5% nonfat dry milk in Tris-buffered saline (TBS). The membranes were then incubated with either monoclonal anti-NOS3 (1:1000 dilution; Transduction Laboratories, Lexington, Kentucky, USA), monoclonal anti-actin (1:1000 dilution; Sigma-Aldrich, St. Louis, Missouri, USA), or polyclonal anti-p-38 (1:3000 dilution; Santa Cruz Biotechnology Inc., Santa Cruz, California, USA) Ab in 5% nonfat dry milk for 1 hour. The p-38 MAP kinase is a highly conserved myocyte protein (34, 35). Actin and p-38 were used to normalize any potential differences in NOS3 abundance. After three washes (20 minutes each) with TBS/0.1% Tween-20, the membrane was incubated for 1 hour with goat anti-rabbit IgG horseradish peroxidase, 1:1000 dilution, in 5% nonfat dry milk. After three washes (20 minutes each) with TBS/0.1% Tween-20, the membrane was exposed to film using chemiluminescence (ECL Western Blotting Detecting Reagents; Calbiochem-Novabiochem). For quantification the films were digitized and densitometry was performed to determine the mean OD of each lane (arbitrary units).

Expression of β_3 -adrenoceptor. RT-PCR was performed using 1 μ g of total RNA (mouse heart and epididymal fat). Total RNA was reverse-transcribed with M-MLV RT (Superscript II) (Life Technologies Inc., Gaithersburg, Maryland, USA) for 30 minutes at 37°C in a solution containing 500 nM oligodT, 0.2 mM of each

dNTP, 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM MgCl₂, and 10 mM dithiothreitol. RNA template was digested with RNase H. Denatured cDNA (for 5 minutes at 94°C) was submitted to 30 cycles of amplification in a solution containing 0.5 U of Taq polymerase (Perkin Elmer, Norwalk, Connecticut, USA) and 1 mM sense and antisense oligonucleotides corresponding to a 340-bp fragment from 651–991 (forward primer, 5′-3′, GTGGGTTGCCAGGAGTACCATG; reverse primer, 5′-3′, TGTCACCAACCTTTGCGT) of the mouse β_3 -adrenoceptor sequence (36). cDNA from mouse hearts and white epididymal fat (as a positive control) were used as a template for the PCR reactions. To further confirm the presence of β_3 transcription, RNase protection was performed using the cloned fragment, as described previously (37). Hearts without reverse transcriptase were used as a control to test for genomic contamination.

Levels of cGMP. FVB ($n = 8$) and $\beta_3^{-/-}$ ($n = 13$) mice were anesthetized and ventilated as described above and infused intravenously with either normal saline or isoproterenol (5 ng/kg/min). Hearts were rapidly excised, homogenized in 6% trichloroacetic acid (1 mL TCA/100 mg tissue), centrifuged, and extracted with water-saturated diethyl ether. The aqueous layer was vacuum dried at -60°C and resuspended in sodium acetate buffer for analysis with an enzyme immunoassay (Amersham Pharmacia Biotech, Piscataway, New Jersey, USA).

Statistics. All results are reported as mean $\pm$ SEM. A one-way ANOVA was used to compare base-line hemodynamics in WT, $\beta_3^{-/-}$, and NOS3 $^{-/-}$ mice. For statistical analysis of isoproterenol concentration-effect relationships, a one-way ANOVA with repeated measures was used to analyze responses per strain. A two-way ANOVA with terms for treatment and strain was used to compare responses between strains. A one-way ANOVA for repeated measures was used to compare the effects of isoproterenol with and without L-NMMA or L-NAME in WT and $\beta_3^{-/-}$ mice. The effects of isoproterenol in NOS3 $^{-/-}$ mice were analyzed with a paired t test. The effects of isoproterenol on cGMP concentration were compared using an unpaired t test with a Bonferroni correction. All statistical analyses were performed using SAS software (Cary, North Carolina, USA). Differences were considered significant at P values less than 0.05.

Results

Effect of β_3 -adrenoceptor and NOS3 deletion on base-line hemodynamic parameters. We used a combined micromanometer-conductance catheter to assess the determinants of cardiovascular performance in WT, $\beta_3^{-/-}$, and NOS3 $^{-/-}$ mice (Table 1). $\beta_3^{-/-}$ mice had similar base-line indices of myocardial contractility and afterload compared with WT mice. Both the $\beta_3^{-/-}$

and NOS3 $^{-/-}$ mice had smaller chamber sizes (lower EDV) than the WT mice, which was accompanied by impaired relaxation (higher tau) in the $\beta_3^{-/-}$ mice. Whereas all three strains of mice had similar body weights, the NOS3 $^{-/-}$ mice had elevated heart weights resulting in increased heart-weight/body-weight ratio. As described previously (23), NOS3 $^{-/-}$ mice had hypertension reflected not only by elevated systemic blood pressure but also by increased Ea, a measure of arterial tone. Also, the NOS3 $^{-/-}$ mice had increased heart rate compared with the WT and $\beta_3^{-/-}$ mice.

Effect of intravenous isoproterenol on myocardial contractility. To assess the β -adrenergic inotropic response we infused isoproterenol (1, 10, 100 ng/kg/min) to anesthetized mice. As shown in Figure 1, isoproterenol produced an augmented contractile response in $\beta_3^{-/-}$ mice ($58 \pm 7.9\%$ increase in dP/dt-IP at peak isoproterenol response; $P < 0.001$) compared with WT mice ($37 \pm 5.9\%$ peak dP/dt-IP increase; $P < 0.001$ vs. base line, $P < 0.01$ vs. $\beta_3^{-/-}$ mice).

The effects on contractility, as assessed by dP/dt-IP, were not related to changes in preload or afterload after isoproterenol infusion, because EDP, EDV, Ea, and systolic blood pressure were not changed after isoproterenol infusion in either WT or $\beta_3^{-/-}$ mice. Heart rate increased similarly in both the $\beta_3^{-/-}$ mice ($21.7 \pm 3.2\%$; $P < 0.01$ vs. base line) and WT mice ($29.0 \pm 4.3\%$; $P < 0.01$ vs. base line, $P =$ not significant [NS] vs. $\beta_3^{-/-}$).

Effect of NOS-inhibition on β -adrenergic stimulation. To determine the role of NO in β -adrenergic contractility we examined the impact of NOS inhibition with L-NMMA on β -adrenergic responses. Mice were administered isoproterenol (5 ng/kg/min) followed by the NOS inhibitor L-NMMA (10 mg/kg/h). At this concentration of isoproterenol, dP/dt-IP increased in both WT ($10.1 \pm 2.6\%$; $P < 0.05$ vs. base line; Figure 2a) and $\beta_3^{-/-}$

Table 1
Base-line conditions

	WT	$\beta_3^{-/-}$	NOS3 $^{-/-}$
<i>n</i>	8	10	15
Heart rate (bpm)	541 $\pm$ 23	559 $\pm$ 15	629 $\pm$ 19 ^A
Body weight (g)	32.4 $\pm$ 0.7	33.7 $\pm$ 0.6	31.4 $\pm$ 2.2
Heart weight (mg)	145 $\pm$ 9	158 $\pm$ 15	192 $\pm$ 9 ^A
Heart-weight/body-weight ratio (mg/g)	4.5 $\pm$ 0.2	4.7 $\pm$ 0.4	6.2 $\pm$ 0.3 ^B
Afterload			
Systolic blood pressure (mmHg)	101 $\pm$ 4	108 $\pm$ 5	132 $\pm$ 5 ^B
Ea (mmHg/ μ L)	6.4 $\pm$ 1.2	8.7 $\pm$ 0.6	16.4 $\pm$ 3.0 ^A
Preload			
EDP (mmHg)	9.9 $\pm$ 1.1	9.4 $\pm$ 0.9	8.7 $\pm$ 0.6
EDV (μ L)	25.9 $\pm$ 4.0 ^B	14.1 $\pm$ 1.0	11.5 $\pm$ 1.6
Contractility			
+dP/dt (mmHg/s)	11,575 $\pm$ 921	11,407 $\pm$ 714	11,639 $\pm$ 675
+dP/dt-IP (s ⁻¹)	194.8 $\pm$ 10.8	184.8 $\pm$ 8.1	162.9 $\pm$ 8.5
Ees (mmHg/ μ L)	12.0 $\pm$ 3.0	16.8 $\pm$ 3.8	15.4 $\pm$ 0.5
Diastole			
Tau (ms)	4.9 $\pm$ 0.2	6.0 $\pm$ 0.4 ^A	5.2 $\pm$ 0.3
-dP/dt (mmHg/s)	-8,915 $\pm$ 836	-8,816 $\pm$ 881	-11,309 $\pm$ 795

Data are reported as mean $\pm$ SEM. ^A $P < 0.05$ vs. other two strains using ANOVA. ^B $P < 0.01$ vs. other two strains using ANOVA.

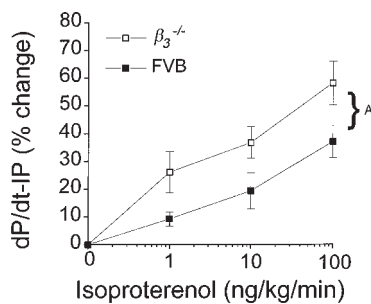


Figure 1

β -Adrenergic concentration-effect curves in $\beta_3^{-/-}$ and WT mice. Isoproterenol was administered intravenously at rates of 1, 10, and 100 ng/kg/min to WT mice (FVB, $n = 12$) and mice with homozygous β_3 -adrenoceptor-deletion mutations ($\beta_3^{-/-}$; $n = 7$). Peak positive dP/dt divided by the instantaneous left-ventricular pressure (dP/dt-IP) was used as an index of contractility and is displayed as a percentage of change from base line. As shown, the inotropic concentration-effect response to isoproterenol was augmented in $\beta_3^{-/-}$ mice, relative to FVB. Each concentration-effect relationship was highly significant by one-way ANOVA ($P < 0.01$). Data are reported as mean $\pm$ SEM. $^A P < 0.01$, $\beta_3^{-/-}$ vs. FVB, by two-way ANOVA.

mice ($24.5 \pm 3.1\%$; $P < 0.001$ vs. base line, $P < 0.001$ vs. WT; Figure 2a). In WT mice, co-infusion of L-NMMA with isoproterenol resulted in an augmentation of the positive inotropic response (dP/dt-IP increased $28.9 \pm 5.6\%$ over base line; $P < 0.01$ vs. base line, $P < 0.05$ vs. isoproterenol alone; Figure 2a). Thus, inhibition of NOS augments β -adrenergic-stimulated inotropy, consistent with a NO-related negative-feedback mechanism over cardiac contractile reserve. In contrast to the WT mice, L-NMMA did not augment the already elevated positive inotropic effect in $\beta_3^{-/-}$ mice (dP/dt-IP increased $26.4 \pm 5.6\%$ over base line; $P < 0.001$ vs. base line, $P = \text{NS}$ vs. isoproterenol alone; Figure 2a). A different NOS inhibitor, L-NAME, was also used in additional experiments. In similar fashion to L-NMMA, L-NAME augmented the isoproterenol inotropic response in WT, but not $\beta_3^{-/-}$, mice (Figure 2b).

We further assessed inotropic responses using the relatively load-independent parameter Ees. As shown in Figure 3, isoproterenol-stimulated increases in Ees were augmented by NOS inhibition with L-NMMA in WT, but not in $\beta_3^{-/-}$, mice.

The contractile effects observed with a co-infusion with L-NMMA were not attributable to changes in preload or afterload (EDV and Ea were not changed). Heart rate increased similarly in the $\beta_3^{-/-}$ and WT mice ($8.2 \pm 2.1\%$ in WT mice and $10.4 \pm 3.9\%$ in $\beta_3^{-/-}$ mice, $P < 0.05$ vs. base line for both groups).

Effect of NOS3 deletion on β -adrenergic stimulation. As an additional control, we tested the effects of isoproterenol in NOS3 $^{-/-}$ mice. Isoproterenol (5 ng/kg/min) infusion caused an increase in dP/dt-IP ($35.2 \pm 6.2\%$; $P < 0.001$ vs. base line; Figure 2a) with a magnitude similar to that observed in $\beta_3^{-/-}$ mice. This observation provides further confirmation of elevated β -adrenergic contractility in mice lacking NOS3.

Concentration of cGMP. To test the impact of altered NO signaling we assessed concentrations of the NO second messenger cGMP. Basal cGMP was elevated in $\beta_3^{-/-}$ (10.7 ± 2 pmol/g, $n = 7$) vs. WT (2.4 ± 0.4 pmol/g, $n = 3$; $P < 0.05$ vs. $\beta_3^{-/-}$). In WT mice, isoproterenol-treated (5 ng/kg/min) hearts had a higher level of cGMP (6.5 ± 0.8 pmol/g, $n = 5$; $P < 0.05$) than hearts treated with normal saline. $\beta_3^{-/-}$ mice did not exhibit increased cGMP with isoproterenol (10.9 ± 1.7 pmol/g with isoproterenol, $n = 6$; $P = \text{NS}$ vs. normal saline).

NOS3 protein abundance. Western blot analysis of myocardial tissue revealed no change in NOS3 protein levels, relative to p-38 or actin, between WT and $\beta_3^{-/-}$ mice (Figure 4a). Protein abundance of NOS3 was similar between WT and $\beta_3^{-/-}$ mice (85 ± 5 vs. 87 ± 3 arbitrary units, $n = 8$ and 9 , respectively). Moreover, when corrected for abundance of either p-38 (1.03 ± 0.06 vs. 1.11 ± 0.10 , $P = \text{NS}$) or actin (0.84 ± 0.08 vs. 1.06 ± 0.13 , $P = \text{NS}$), NOS3 was similarly unchanged. Whereas NOS3 protein was absent in the NOS3 $^{-/-}$ mice, expression of p-38 and actin was present in similar quantities to the other strains of mice (Figure 4a).

Expression of β_3 -adrenoceptor. Expression of β_3 -adrenoceptor mRNA was detected by RT-PCR in heart and epididymal fat of both WT and NOS3 $^{-/-}$ mice, but not $\beta_3^{-/-}$ animals (Figure 4b). RT-PCR in reverse-transcribed negative reactions revealed little or no PCR product, confirming minimal contamination with genomic DNA. RNase protection assay confirmed the presence of β_3 -adrenoceptor mRNA in WT and NOS3 $^{-/-}$ mice (Figure 4c).

Discussion

In this study we have shown that mice lacking the β_3 -adrenoceptor do not exhibit NO-mediated inhibition of

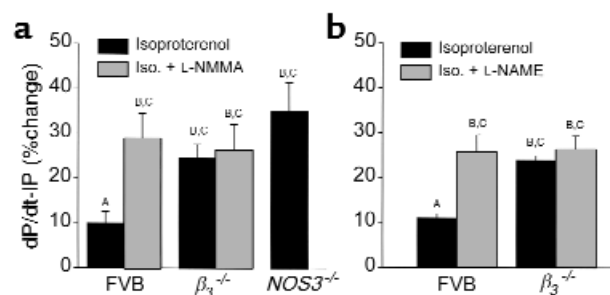


Figure 2

(a) Contractile effects of isoproterenol and L-NMMA in WT, $\beta_3^{-/-}$, and NOS3 $^{-/-}$ mice. Isoproterenol was administered intravenously at a rate of 5 ng/kg/min for 4 minutes, followed by a co-infusion with L-NMMA at 10 mg/kg/h for 5 minutes. Contractility was indexed by dP/dt-IP and is shown as a percentage of change from base line. The $\beta_3^{-/-}$ mice ($n = 10$) had greater responses to isoproterenol than did the WT mice ($n = 8$), but did not show any further augmentation after NOS inhibition with L-NMMA. Similarly, NOS3 $^{-/-}$ mice ($n = 15$) were hyper-responsive to isoproterenol. L-NMMA augmented the isoproterenol response in WT mice to the level observed in $\beta_3^{-/-}$ mice. (b) The effect of an additional NOS inhibitor, L-NAME. L-NAME had an effect similar to L-NMMA, augmenting the response to isoproterenol in WT, but not $\beta_3^{-/-}$, mice. Data are reported as mean $\pm$ SEM. $^A P < 0.05$ vs. respective base line; $^B P < 0.01$ vs. respective base line; $^C P < 0.05$ vs. WT isoproterenol response by one-way ANOVA.

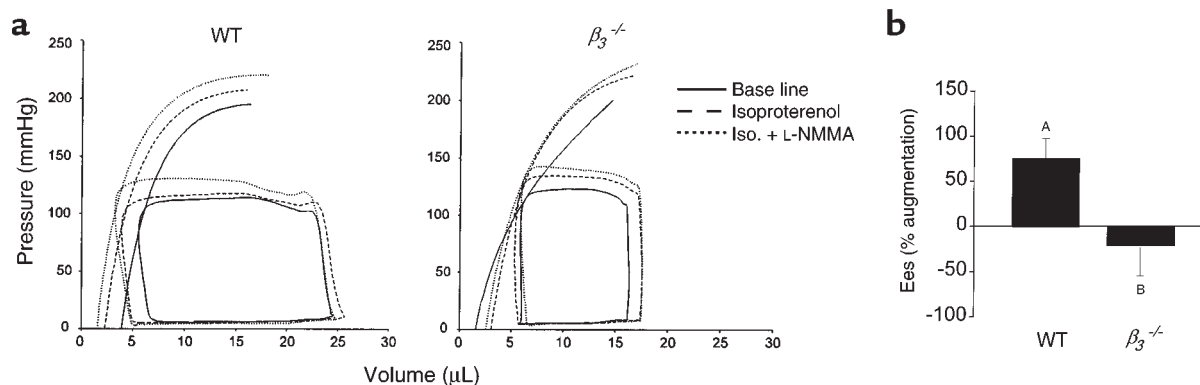


Figure 3

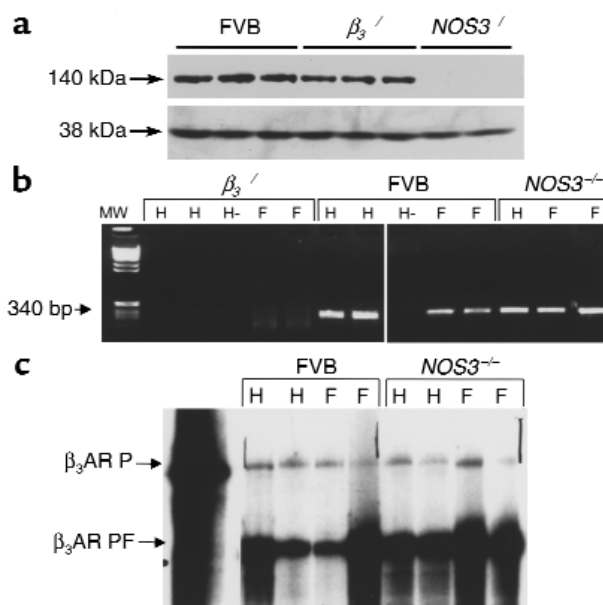
Left ventricular pressure-volume data in WT and $\beta_3^{-/-}$ mice. A combined micromanometer-conductance catheter was inserted into the LV through the apex. Transient occlusion of the descending aorta was used to generate the end-systolic pressure-volume relationship (loops not shown). Depicted are (a) example steady-state loops and their respective ESPVR (from which Ees is determined) at base line after receiving isoproterenol (5 ng/kg/min) and after receiving isoproterenol and L-NMMA (10 mg/kg/h). Also shown is (b) pooled data of the augmentation of isoproterenol-stimulated inotropy by L-NMMA in WT ($n = 8$) and $\beta_3^{-/-}$ ($n = 10$) mice. Isoproterenol-induced increases in Ees were augmented by NOS inhibition in WT, but not in $\beta_3^{-/-}$ mice. Data are reported as mean $\pm$ SEM. ^A $P < 0.05$ vs. base line by paired t test; ^B $P < 0.05$ vs. WT by unpaired t test.

β -adrenergic-stimulated inotropic responses. The cardiovascular effects of β_3 -adrenoceptor deletion in mice were apparent only during sympathetic stimulation. Under base-line conditions, the $\beta_3^{-/-}$ and WT mice exhibited similar heart rates, loading conditions, and contractile states. However, when stimulated with isoproterenol, a nonspecific β -adrenergic agonist, the $\beta_3^{-/-}$ mice had a greater positive inotropic response than did the WT mice. In WT mice the inotropic response to isoproterenol was augmented by L-NMMA and L-NAME, NOS inhibitors, to the level seen in $\beta_3^{-/-}$ mice with isoproterenol alone. Augmentation by L-NMMA or L-NAME was absent in the $\beta_3^{-/-}$ mice. Similarly, mice lacking *NOS3* were hyper-responsive to isoproterenol. Taken together, these observations suggest that the β_3 -adrenoceptor plays a major role in NO attenuation of β -adrenergic-stimulated positive inotropy.

These present findings agree with those of Gauthier et al. (12, 13, 19) that direct stimulation of β_3 -adrenoceptors produces a negative inotropic response due to NO-cGMP signaling. These previous studies were unable to address the impact of inhibiting the β_3 -NO pathway because of the lack of availability of β_3 -specific antagonists. The present study, by examining the β_3 -NO pathway in animals lacking the β_3 -adrenoceptor, has established the importance of this receptor in NO-mediated negative feedback over β -adrenergic inotropic responses. In WT mice, NOS inhibition augments β -adrenergic-stimulated positive inotropic effects (2, 3). $\beta_3^{-/-}$ mice, on the other hand, are hyper-responsive to isoproterenol infusion, lack augmentation with NOS inhibition, and yet have similar indices at base line. These observations suggest that β_3 -adrenoceptor

Figure 4

Abundance of NOS3 protein and $\beta_3^{-/-}$ mRNA in myocardium. (a) Western blot of NOS3 from mouse heart tissue. Equal amounts of protein extracts were resolved on agarose gels, transferred to nitrocellulose, and exposed to anti-NOS3 Ab or anti-p-38 Ab. The FVB and $\beta_3^{-/-}$ mice had similar NOS3 abundance relative to p-38 MAP kinase, and NOS3 was absent in the *NOS3*^{-/-} mice. (b) Representative ethidium-stained agarose gel demonstrating expression of β_3 -adrenoceptor mRNA in different mice strains: mRNA is expressed in both heart (H) and epididymal fat (F) of *NOS3*^{-/-} and FVB control mice, but not $\beta_3^{-/-}$ mice. Little or no PCR product is amplified in reactions lacking reverse transcriptase (H-), confirming minimal genomic contamination of mRNA. (c) Autoradiograph of RNase protection assay confirming the expression of β_3 -adrenoceptor in the myocardium of FVB and *NOS3*^{-/-} mouse hearts. β_3 AR P, β_3 -adrenoceptor probe; β_3 AR PF, β_3 -adrenoceptor-protected fragment.



stimulation is coupled to NOS activation, that this coupling offers significant negative feedback over sympathetic activation, and that the β_3 -adrenoceptor exerts its influence primarily during sympathetic activation.

$\beta_3^{-/-}$ animals had elevated myocardial cGMP levels compared with FVB controls, but only WT animals demonstrated increased cGMP concentrations with exposure to β -adrenergic agonists. The former suggests that the β_3 -adrenoceptor negatively modulates basal NO-cGMP production and is consistent with several studies showing that receptors that are coupled to NOS inhibit its activity by a mechanism involving caveolin, the scaffolding protein found in membrane caveolae (see ref. 38 for review and ref. 4). Alternatively, it must be considered that other pathways capable of stimulating cGMP production (e.g., atrial natriuretic peptides; ANP) may be upregulated in these animals. In this regard, Gyurko and colleagues have demonstrated recently increased ANP in *NOS3*^{-/-} mice, which results in cGMP levels similar to WT controls (5). The observation that isoproterenol increases cGMP is consistent with β -agonist linkage to NO resulting in cGMP elevation (11). The absence of this response in $\beta_3^{-/-}$ is further evidence of a signaling linkage between the β_3 -adrenoceptor and NOS.

Sympathetic activation of the β -adrenergic pathway in the heart produces positive inotropic, chronotropic, and lusitropic responses. These responses are mediated through the activation of the β_1 - and β_2 -adrenoceptors coupled to a stimulatory G protein (G_s) (39), which in turn stimulates cAMP production by adenylyl cyclase (AC) (39). cAMP, through cAMP-dependent protein kinase-A (PKA), directly augments the L-type Ca^{++} current (I_{Ca}), which enhances excitation-contraction coupling (40). The best-understood signaling pathway opposing β -adrenergic cardiac activation is mediated by muscarinic-cholinergic stimulation of the heart (41, 42). Muscarinic receptors are coupled to an inhibitory G protein ($G_{i\alpha}$) that both inhibits AC production of cAMP and stimulates NO production via *NOS3* (42). NO, which stimulates the production of cGMP, can have both cGMP-dependent (43–47) and independent (48–50) inotropic effects. Thus, the traditional view of cardiac inotropic regulation by the autonomic nervous system is that of two limbs of the autonomic nervous system acting through separate receptor/signaling pathways, exerting opposing forces on myocardial regulation.

Description of β_3 -adrenoceptors in the heart (13) activated by traditional β -agonists, leading to negative inotropic effects, has raised the possibility that there may be negative feedback over contractility within the sympathetic pathway itself. Initially, β_3 -adrenoceptors were found to be expressed in adipose tissues, where their activation promotes lipolysis and energy expenditure, and in gastrointestinal tissues (16–18), where they regulate motility. Recently, unique β_3 -selective agonists have been shown to cause negative inotropic effects in the heart (12, 13, 19, 51) that are accompanied by increases in NO and production of cGMP (12). Inhibition of NO attenuates the negative inotropic effects of

β_3 -selective agonists (12), suggesting that β_3 -adrenoceptor activation is coupled to NOS stimulation. Activation of NOS by the β_3 -adrenergic pathway may be through a $G_{i\alpha}$ protein, similar to the muscarinic system (42), since the negative inotropic effects of β_3 -adrenergic stimulation are attenuated by pertussis-toxin treatment (12, 13). Unlike the muscarinic-cholinergic pathway, the β_3 pathway may serve as negative feedback over sympathetic activation as β_3 receptors are activated by the same agonists that activate $\beta_{1,2}$ -adrenoceptors. These findings suggest a role for the β_3 -adrenoceptor in inhibition of $\beta_{1,2}$ -adrenergic-stimulated positive inotropic effects in a pathway that is NO mediated.

Recent studies demonstrating that the β_3 -adrenoceptor is upregulated in humans (52) and animals (53) with heart failure (52) suggest that the β_3 -NO pathway has implications in the pathophysiology of heart failure. The failing heart is characterized by downregulation of both the β_1 - and β_2 -adrenoceptors (54), through protein kinase A or β -adrenoceptor kinase (55), and subsequent attenuation of catecholamine induced positive inotropy. Unlike the $\beta_{1,2}$ subtypes, the β_3 -adrenoceptor is unlikely to be downregulated by these mechanisms (56). Given the high sympathetic tone associated with heart failure, the increased ratio of β_3 - to $\beta_{1,2}$ -adrenoceptors and their respective inotropic effects could have profound consequences important for myocardial function in the failing heart.

This study uses a miniature combined manometer-conductance catheter to acquire pressure-volume loops in mice, thus enabling integrated measurement of cardiovascular performance. This method allows for the simultaneous determination of load-independent measures of contractility (Ees, for example), preload, afterload, and heart rate. The combined conductance-manometer catheter permits accurate determination of cardiovascular performance and has been used to characterize cardiomyopathy in transgenic mice (24, 57). Use of this technique has also shown that $\beta_3^{-/-}$ and *NOS3*^{-/-} mice have smaller chamber sizes than WT mice. The latter can be attributed to myocardial hypertrophy (increased heart-weight/body-weight ratio), whereas the former appears to be related to diastolic filling abnormalities (increased tau). The pressure-volume data illustrate that these differences do not influence the contractile parameters reported here (i.e., Ees and dP/dt-IP are independent of chamber size). Diastolic abnormalities mediated by the β_3 -NO pathway are under active investigation by our laboratory.

In conclusion, we have established a physiologic role for the β_3 -adrenoceptor in the heart. Using transgenic mice lacking the β_3 -adrenoceptor, we have shown that the β_3 -adrenoceptor is responsible for NO-mediated negative feedback over β -adrenergic-stimulated positive inotropy.

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