Supplementary Information for

The β-arrestin biased β-adrenergic receptor blocker carvedilol enhances skeletal muscle contractility.

Jihee Kim[a1], Chad A. Grotegut[b1], James W. Wisler[a], Lan Mao[c], Paul B. Rosenberg[a], Howard A. Rockman[ade], and Robert J. Lefkowitz[afg2]

Robert J. Lefkowitz
Email: lefko001@receptor-biol.duke.edu

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Supplementary Information

Materials and Methods

Immunohistochemistry

EDL muscles were isolated after 4 weeks of drug delivery and mounted in O.C.T. (optimal cutting embedding medium) with gum tragacanth (Sigma, at a 4:1 ratio), and flash frozen in an isopentane bath suspended in liquid nitrogen. Cross sections of muscle were cut from the belly of the muscle at a thickness of 10 μm. Sections were fixed with ice-cold acetone for 10 min. For myofiber counts and cell size determination, a simple multicolor immunofluorescence procedure (1) was performed with primary antibodies against MHC I (BA-F8, 1:250 dilution), MHC IIa (SC-71, 2F7), MHC IIb (BF-F3), and polyclonal dystrophin (1:1,000 dilution). Staining was visualized simultaneously using Alexa Fluor 488 anti-mouse IgG2a (BF-F3, MHC IIb), Alexa Fluor 568 anti-mouse IgG1 (SC-71, MHC IIa), Alexa Fluor 647 anti-mouse IgG2b (BA-F8, MHC I), and Alexa Fluor 405 anti-rabbit (dystrophin). Primary antibodies (BF-F3, SC-71, and BA-F8) were purchased from the Developmental Studies Hybridoma Bank (University of Iowa), anti-dystrophin antibody (Sigma-Aldrich), and secondary antibodies were purchased from Invitrogen. All sections were mounted in Vectashield (Vector labs). Slides were visualized with a Zeiss LSM510 laser scanning microscope (3i) using conventional wide field fluorescence microscopy. Individual images were taken across the entire cross-section and assembled into a composite montage image with Slidebook 6 program (3i). For fiber type analysis, all fibers within the entire muscle/cross-section were examined in a blind manner. Fiber cross sectional area (CSA) was measured for each fiber type by outlining at least 40% of all fibers within a muscle/cross-section using Image J program (NIH). Fiber type percentages, and fiber CSA data are reported as group means ± SEM based on individual animal. The mean response for each treatment group was compared using a one-way ANOVA and the Student's *t*- test (Prism).

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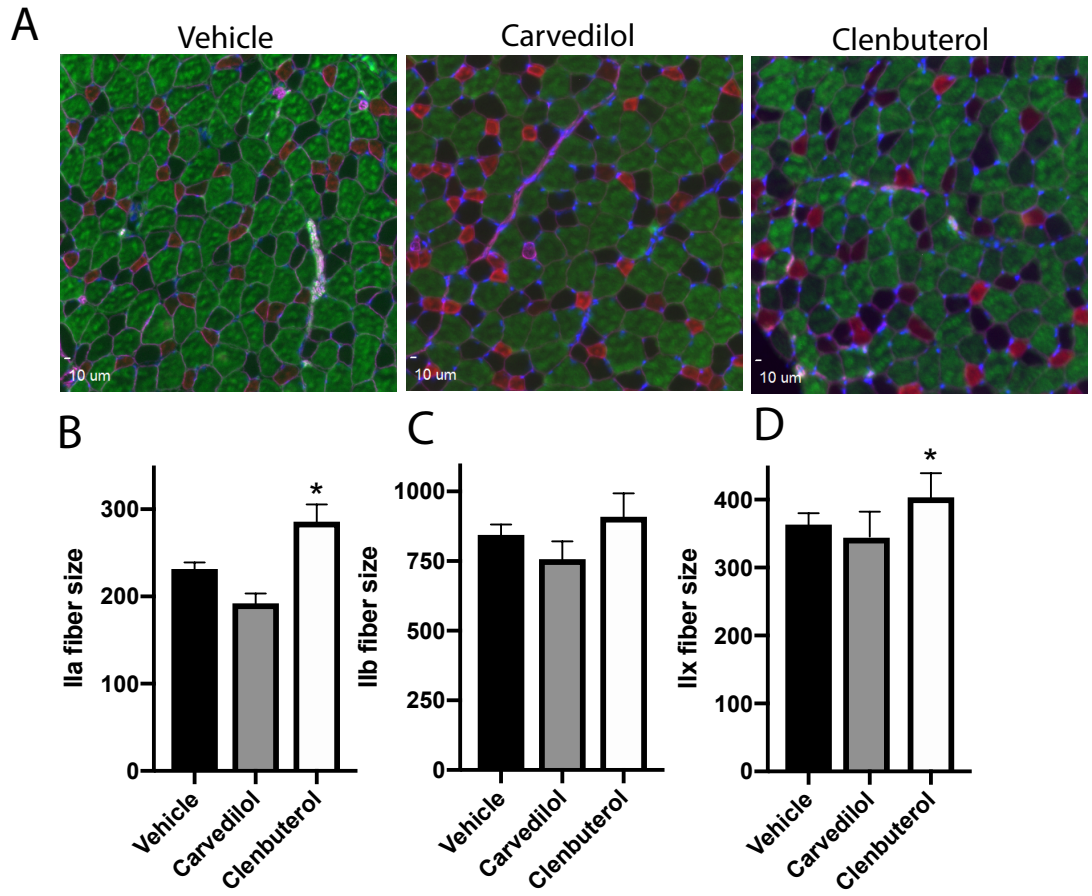

Figure S1. **Clenbuterol and carvedilol affected on fiber size change**. (a) Representative immunohistochemistry images of MHC isoforms were presented from EDL muscles from βarr1flox mice treated by vehicle, clenbuterol (1mg/kg/day), and carvedilol (1mg/kg/day) for four weeks. Multicolor immunohistochemistry was performed with primary antibodies against MHC I (BA-F8), MHC IIa (SC-71, 2F7), MHC IIb (BF-F3), and polyclonal dystrophin. Staining was visualized simultaneously using Alexa Fluor 488 anti-mouse IgG2a (BF-F3, MHC IIb, Green), Alexa Fluor 568 anti-mouse IgG1 (SC-71, MHC IIa, Red), Alexa Fluor 647 anti-mouse IgG2b (BA-F8, MHC I, Purple), and Alexa Fluor 405 anti-rabbit (dystrophin, Blue). Fiber size of MHC IIa, IIb, and IIx was measured and presented as a fold increase over vehicle stimulation for IIa (B), IIb (C), and IIx (D). Data shown represent the means ± SEM from 3-4 mice per group. Statistical comparison was performed by using a one-way ANOVA and the Student's *t*- test (Prism) (*, P< 0.05).

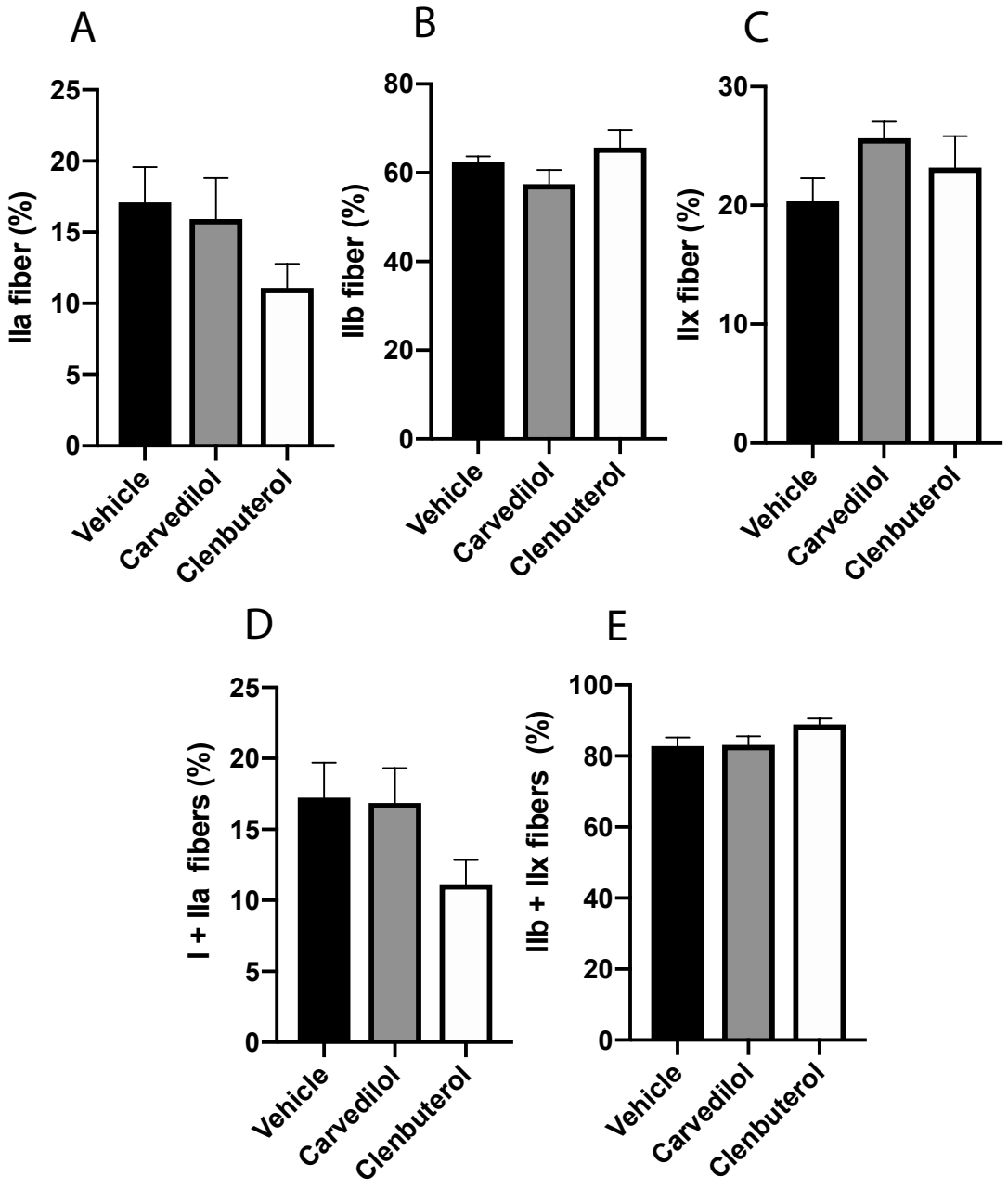

Figure S2. **Effect of clenbuterol and carvedilol on skeletal muscle fiber composition of EDL.** Quantification of fibers from the immunohistochemistry images from clenbuterol or carvedilol treated EDL muscles. (A) Percentage of type IIa fibers, (B) percentage of IIb fibers, (C) Percentage of IIx fibers. (D) Percentage of fiber combining type I and IIa fibers. (E) Percentage of fibers combining type IIb and IIx fibers. Data shown represent the means ± SEM from 3-4 mice per group.

Table S1. **The effect of β-blockers on EDL muscle contraction in β-arrestin 1 flox and skeletal muscle specific knockout mice.** Values are means ± standard error (S.E.). Statistical analysis was performed using a one-way ANOVA with Dunnett's multiple comparison test (*, P < 0.05, **, P < 0.01, ***, P <0.001). P values were compared to its vehicle treatment control. Statistical analysis was performed using a two-way ANOVA with Sidak's multiple comparison test (#, P < 0.05, ##, P < 0.01, ###, P <0.001) compared between βarr1Flox and βarr1smKO under the same drug treatment described in materials and methods.

Drug	**Vehicle**		**Clenbuterol**		**Carvedilol**		**Metoprolol**		**Nadolol**	**Carvedilol + Nadolol**
Genotype	**βarr1Flox**	**βarr1smKO**	**βarr1Flox**	**βarr1smKO**	**βarr1Flox**	**βarr1smKO**	**βarr1Flox**	**βarr1smKO**	**βarr1Flox**	**βarr1Flox**
Body weight (g)	28.0 ± 1.0	28.7 ± 1.0	29.9 ± 1.0	29.7 ± 0.6	24.8 ± 0.6 *	27.4 ± 0.7	31.8 ± 1.3 *	30.7 ± 0.4	26.9 ± 0.5	27.3 ± 0.6
Wet Muscle weight, MW (mg)	9.2 ± 0.3	8.5 ± 0.5	11.0 ± 0.5 *	9.5 ± 0.3	7.4 ± 0.2 **	7.5 ± 0.3	9.3 ± 0.5	8.6 ± 0.4	9.2 ± 0.5	9.2 ± 0.3
Dry Muscle weight, MW (mg)	2.3 ± 0.2	2.1 ± 0.2	2.4 ± 0.1	2.4 ± 0.1	1.6 ± 0.2	1.7 ± 0.2	1.6 ± 0.1	1.8 ± 0.1	2.1 ± 0.1	2.4 ± 0.2
Tibia length, TL (cm)	1.8 ± 0.0	1.8 ± 0.0	1.8 ± 0.0	1.8 ± 0.0	1.7 ± 0.0	1.8 ± 0.0	1.8 ± 0.0	1.8 ± 0.0	1.8 ± 0.0	1.8 ± 0.0
MW/TL (mg/cm)	5.2 ± 0.2	4.7 ± 0.3	6.1 ± 0.2 *	5.3 ± 0.1 ##	4.3 ± 0.2 **	4.2 ± 0.2	5.1 ± 0.3	4.8 ± 0.2	5.2 ± 0.2	5.2 ± 0.2
EDL Length (mm)	10.5 ± 0.1	10.6 ± 0.0	10.8 ± 0.2	10.5 ± 0.1	10.1 ± 0.1	10.5 ± 0.2	10.1 ± 0.1	10.3 ± 0.1	10.0 ± 0.2	10.5 ± 0.1
EDL Width (mm)	1.2 ± 0.1	1.4 ± 0.1	1.1 ± 0.00	1.3 ± 0.1	1.0 ± 0.1	0.9 ± 0.2	1.2 ± 0.1	1.3 ± 0.1	1.1 ± 0.1	1.2 ± 0.1
Xsec area (mm2)	1.2 ± 0.2	1.7 ± 0.2	0.9 ± 0.1	1.3 ± 0.2	0.8 ± 0.1	0.7 ± 0.1	1.1 ± 0.1	1.3 ± 0.2	1.0 ± 0.1	1.2 ± 0.1
Twitch force (mN)	37.9 ± 2.7	35.4 ± 4.6	50.4 ± 3.5 *	31.5 ± 2.7 ##	32.9 ± 2.6	24.6 ± 2.4	32.9 ± 3.4	33.4 ± 4.7	33.6 ± 3.7	38.5 ± 2.0
Force at 160Hz (mN)	146.3 ± 9.8	134.6 ± 18.1	226.1 ± 29.4 ***	119.1 ± 17.5 ###	163.6 ± 14.5	79.4 ± 7.9 ###	103.0 ± 15.4	109.9 ± 16.4	75.5 ± 5.3 ***	118.3 ± 10.0
Force at 300Hz (mN)	107.6 ± 8.2	93.3 ± 5.5	185.9 ± 28.1 **	113.4 ± 13.2 ##	156.6 ± 15.8 *	65.7 ± 7.4 ###	81.5 ± 15.5	89.5 ± 14.2	58.7 ± 7.2 **	100.5 ± 12.3
Fatigue time 1/2 train (sec)	77.3 ± 3.2	73.5 ± 5.2	68.6 ± 1.7	76.3 ± 4.1	76.1 ± 4.1	68.2 ± 3.2	72.8 ± 4.1	72.3 ± 1.6	100.5 ± 3.9 ***	92.7 ± 3.3 **
Age (days)	118 ± 9	114 ± 19	103 ± 11	95 ± 1	99 ± 10	107± 19	140 ± 9	140 ± 10	92 ± 0	91 ± 11
N	8	5	5	7	10	8	6	5	5	9

Dataset S1. **The effect of β-blockers on EDL muscle contraction in β-arrestin 1 flox mice.**

Dataset S2. **The effect of β-blockers on EDL muscle contraction in skeletal muscle specific β-arrestin 1 knockout mice.**

References

1. D. Bloemberg, J. Quadrilatero, Rapid determination of myosin heavy chain expression in rat, mouse, and human skeletal muscle using multicolor immunofluorescence analysis. *PLoS One* **7**, e35273 (2012).