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CHRONIC ORAL ADMINISTRATION OF ANG-(1-7) IMPROVES SKELETAL MUSCLE, AUTONOMIC, AND LOCOMOTOR PHENOTYPES IN MUSCULAR DYSTROPHY

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Abstract

Muscular dystrophies are a group of heterogeneous genetic disorders that cause progressive muscle weakness and wasting, dilated cardiomyopathy and early mortality. There are different types of muscular dystrophies with varying etiologies but they all have a common hallmark of myofiber degeneration, atrophy and decreased mobility. Mutation in sarcoglycan-delta (Sgcd), a subunit of dystrophin glycoprotein complex, causes Limb Girdle Muscular Dystrophy 2F (LGMD2F). Previously we have reported that Sgcd deficient (Sgcd^{-/-}) mice exhibit angiotensin II (Ang II) induced autonomic and skeletal muscle dysfunction at a young age which contributes to onset of dilated cardiomyopathy and mortality at older ages. Two counter-regulatory renin angiotensin system (RAS) pathways have been identified – deleterious actions of angiotensin II (Ang II) acting on type 1 receptor (AT₁R) versus protective actions of Ang-(1-7) acting on Mas receptors. We propose that the balance between the Ang II/AT₁R and Ang-(1-7)/Mas axes is disturbed in Sgcd^{-/-} mice. Control C57BL/6 and Sgcd^{-/-} mice were treated with Ang-(1-7) included in hydroxypropyl β -cyclodextrin (drinking water) for 8–9 weeks beginning at 3 weeks of age. Ang-(1-7) treatment restored Ang II/AT₁R vs. Ang-(1-7)/Mas balance, decreased oxidative

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AUTHOR CONTRIBUTIONS

Rasna Sabharwal conceived and designed the study, performed the experiments, analyzed the data, interpreted the results and wrote the paper. Michael Cicha carried out Western blot analysis. Robson Santos and collaborators from UFMG provided the CD-Ang-(1-7) compound used in this study. Mark Chapleau was involved in interpretation of results and wrote the paper. All authors have read and approved the final paper.

DISCLOSURES

Drs. Sabharwal, Santos and Chapleau are collaborating with Tarix Pharmaceuticals in developing novel therapies for muscular dystrophy.

stress and fibrosis in skeletal muscle, increased locomotor activity, and prevented autonomic dysfunction without lowering blood pressure in *Sgcd*^{-/-} mice. Our results suggest that correcting the early autonomic dysregulation by administering Ang(1-7) or enhancing its endogenous production may provide a novel therapeutic approach in muscular dystrophy.

Keywords

sarcoglycans; renin angiotensin system; dystrophic glycoprotein complex; autonomic regulation; neuromuscular disorders; ACE/AngII/AT₁R axis; ACE2/Ang1-7/Mas axis

INTRODUCTION

Sarcoglycans are members of the dystrophin glycoprotein complex (DGC) found in the sarcolemma that are important in maintaining integrity of the muscle against forces generated during contractions [1, 2]. Mutation of sarcoglycan-delta (*Sgcd*) causes limb girdle muscular dystrophy 2F (LGMD2F), characterized by progressive wasting of muscle and weakness, and dilated cardiomyopathy (DCM) in both humans and animals [3].

There is evidence of aberrant neurohumoral activation in muscular dystrophy [4, 5]. Amongst the numerous neurohumoral systems involved in cardiovascular homeostasis, the reninangiotensin system (RAS) is of paramount importance. Angiotensin converting enzyme (ACE) converts angiotensin I to angiotensin II (Ang II) that acts *via* angiotensin type 1 (AT₁R) and type 2 (AT₂R) receptors. Pathogenesis of a disease state due to the actions of RAS is primarily mediated by the Ang II/AT₁R axis [6]. A powerful counter-regulatory RAS axis has been described recently [7, 8]. Ang II is cleaved by angiotensin converting enzyme 2 (ACE2) to form the heptapeptide angiotensin-(1-7) (Ang-(1-7)) that acts *via* the Mas receptors (Mas). Ang-(1-7) is an endogenous ligand for Mas [9]. Ang-(1-7) has been demonstrated to possess anti-fibrotic, anti-remodeling, antioxidant, sympathoinhibitory and vasodilatory properties [10, 11].

Recently, we demonstrated that *Sgcd*^{-/-} mice exhibit severe autonomic and skeletal muscle dysfunction at a young age that worsens with aging, and contributes to DCM and early mortality (12, 13). This early-age dysregulation in *Sgcd*^{-/-} mice is mediated by deleterious actions of Ang II binding to AT₁R; the dysregulation was essentially abolished by chronic treatment with the AT₁R antagonist losartan (13). Expression of AT₁R and ACE activity are augmented in muscular dystrophy [13–15]. Activation of the ACE/Ang II/AT₁R axis induces skeletal muscle fibrosis in muscular dystrophy by increasing transforming growth factor type β 1 (TGF β 1) and connective tissue growth factor [15, 16].

Most current therapies and translational approaches have focused on reversing skeletal muscle fibrosis and pathology, with relatively little attention to correcting the impaired autonomic regulation in muscular dystrophy. In this study, we hypothesized that chronic administration of Ang-(1-7) will counterbalance the deleterious actions of Ang II to prevent autonomic and skeletal muscle dysfunction in young *Sgcd*^{-/-} mice.

MATERIALS AND METHODS

Animals

Experiments were performed on age-matched young (10-13 wks) control C57BL/6 and *Sgcd*^{-/-} mice. Generation of homozygous *Sgcd*^{-/-} mice has been described previously [2]. The mice were maintained in a 12:12 hr light-dark cycle (6:00 AM to 6:00 PM), fed normal mouse chow, and had access to water *ad libitum*. All procedures were performed in accordance with American Physiological Society and institutional guidelines.

Experimental Groups

At 3 weeks of age, control and *Sgcd*^{-/-} mice pups were randomly assigned into four groups - untreated control, Ang-(1-7)-treated control, untreated *Sgcd*^{-/-} and Ang-(1-7)-treated *Sgcd*^{-/-} mice. The number of male and female mice in each group was balanced. Ang-(1-7) was included in hydroxypropyl β -cyclodextrin (CD-Ang-(1-7)) to ensure stability of the drug [17]. The formulation was administered in the drinking water (10 μ g/ml) to the treated groups beginning at 3 weeks of age for 8–9 weeks.

Assessment of Blood Pressure, Heart Rate, Autonomic Regulation and Locomotor Activity

At 8–9 weeks of age, a radiotelemetry probe (PC10, DSI) was chronically implanted into the thoracic aorta *via* the left common carotid artery in mice anesthetized with ketamine and xylazine (91 μ g/g and 9.1 μ g/g, respectively, IP) as described previously [18, 19]. After one week of recovery from the implantation surgery, arterial blood pressure (BP), heart rate (HR), and locomotor activity were measured over three days using Dataquest ART Acquisition software [18, 19]. HR was derived from measurements of the arterial pulse intervals. In addition, BP was recorded continuously (2000 Hz) for one hour to collect beat-to-beat data for assessment of spontaneous baroreflex sensitivity (BRS), cardiac vagal and sympathetic tone, and vasomotor sympathetic tone [18, 19]. Resting cardiac vagal and sympathetic tones were measured as changes in HR in response to the muscarinic cholinergic receptor blocker methylatropine (1 mg/kg, IP; Sigma) and the β -adrenergic receptor blocker propranolol (1 mg/kg, IP; Sigma), respectively, while resting vasomotor sympathetic tone was measured as the change in mean BP in response to the ganglionic blocker chlorisondamine (12 μ g/g, IP; Tocris) [18, 19]. Responses to pharmacological drugs were measured when mice were inactive, as determined by the locomotor activity trace. BRS was calculated from spontaneous fluctuations in systolic BP and HR measured when the mice were active using the sequence technique [18, 19]. Measurements of spontaneous locomotor activity were derived from the changes in transmitter signal strength associated with movement of the mouse. All these measurements were made in untreated and Ang-(1-7)-treated control and *Sgcd*^{-/-} mice at a young (10–13 weeks) age.

Measurement of Gene Expression, Fibrosis and Oxidative Stress in Skeletal Muscle

At the end of the study mice were anesthetized with sodium pentobarbital (1.5 mg/10 g of body weight, IP) to prepare skeletal muscle samples (quadriceps). 50% of the mice were perfused with 15 ml of PBS and zinc formalin fixative solution for embedding tissues in paraffin, while frozen muscle samples were prepared from the remaining mice.

(i) Fibrosis—Paraffin blocks of quadriceps were cut into 7 μm sections and stained with freshly prepared Masson's Trichrome. Masson Trichrome stains nuclei black, cytoplasm in muscle fibers red, and collagen blue. The stained sections were imaged with an Olympus BX-51 Light Microscope equipped with a DP-71 digital camera. The images were printed onto paper and overlaid with a transparency imprinted with a counting grid containing points evenly spaced according to the rules outlined previously [20]. The space between these course points was used to calculate area of fibrosis. This was done for each section stained. Six to eight sections from each mouse were analyzed, and the average percent fibrosis was calculated for each mouse. In addition, muscle sections were stained with primary antibodies for collagen III (Abcam #ab7778) or fibronectin (Abcam #ab6328) and hematoxylin, and followed by detection with chromagen anti-Mouse HRP/DAB kit (Vector #SK4100). The reagents in the HRP/DAB (horseradish peroxidase/3,3'-diaminobenzidine) kit constitute a labeled streptavidin-biotin immunoenzymatic antigen detection system that involves sequential incubation of the specimen with primary antibody specific to the target antigen, a biotinylated secondary antibody, enzyme-labeled streptavidin, and substrate-chromogen. The enzymatic reaction produces a dark brown color. The stained sections were imaged with an Olympus BX-51 Light Microscope equipped with a DP-71 digital camera.

(ii) Immunofluorescence—For immunostaining, the tissues were embedded with OCT compound and rapidly frozen in isopropanol-chilled liquid nitrogen. They were stored at -80°C until 7 μm sections were made using a cryostat. The primary antibody for detection of AT₁R-immunoreactivity was a rabbit polyclonal antibody to the AT₁R (1:100 dilution; catalog no. sc-1173, Santa Cruz). Mas-immunoreactivity was detected using anti-Mas antibody (1:60 dilution; AAR-013 Alomone Labs). The sections were incubated with a secondary antibody, Alexa Fluor 488 goat, anti-rabbit IgG and DAPI to counterstain cell nuclei. Immunostaining was visualized with a confocal microscope (Zeiss 710).

(iii) Oxidative Stress—Superoxide ($\text{O}_2^{\bullet-}$) levels measured by dihydroethidium (DHE, Sigma) fluorescence were used as a marker of oxidative stress in skeletal muscle. DHE stock solution (20 nM) was prepared by dissolving DHE (catalog D1168, Invitrogen) in dimethylsulphoxide (DMSO) under argon vapor. 8 μl of the DHE stock solution was diluted into 20 ml of PBS which was then applied onto quadriceps sections for 15 min at 37°C and protected from light. Images were captured on a confocal microscope (BioRad 1024) at excitation 488 nm and emission wavelength of 568 nm. An average of six (7 μm) sections of skeletal muscle were obtained from each mouse. Intensity of fluorescence was quantified using NIH ImageJ software and normalized to a percentage of the average fluorescence intensity measured in untreated control mice.

(iv) Western Blot—For Western blot analysis of AT₁R and Mas protein expression, quadriceps muscles were directly snap frozen without OCT and stored at -80°C . The samples were subsequently ground on a mortar and pestle. The tissue was added to 500 μl of homogenization buffer (100 mM Hepes, 320 mM sucrose, 1 mM PMSF with 1% protein inhibitor cocktail (Sigma P8340) and given twenty strokes in a glass dounce homogenizer. The sample was spun at 4000 g for 10 min. The supernatant was removed, and the tissue was resuspended in 500 μl of buffer and spun again. The pooled supernatants were spun at

100,000g for 60 minutes at 4 degrees C. The supernatant was removed and the separated membrane fraction was suspended in 250 ul of homogenization buffer. Protein content was determined using a Bradford protein assay (Bio-Rad). The membranes were blocked in Tris-Buffered Saline containing 0.1 % Tween-20 (TBS-T) with 5 % non-fat dried milk for 3 hours at room temperature, then incubated overnight at 4 degrees C in anti-AT₁R antibody (sc-1173, Santa Cruz) diluted 1:5000 in TBS-T with 3 % BSA. For Mas receptors, we used anti-Ang-(1-7) Mas receptor antibody (AAR-013, Alomone Labs) diluted 1:7500 in TBS-T with 3 % BSA. The membranes were rinsed and then incubated in peroxidase-labeled goat-anti-rabbit IgG (Jackson) diluted 1:120,000 in TBS-T. The bands were visualized with an ECL reagent (Amersham). The membranes were then stripped, blocked, and incubated with peroxidase-labeled anti-GAPDH (NB-110, Novus Bio) diluted 1:250,000 in TBS-T with 3 % BSA, and visualized with ECL as a loading control. The films were scanned and analyzed using NIH ImageJ software.

Data Analysis

The results are expressed as means $\pm$ SEM. Significant differences were defined at $P < 0.05$. Statistical evaluation was performed using unpaired t-test to compare between experimental groups (StatView SAS Institute, Cary, NC).

RESULTS

CD-Ang-(1-7) Prevents Autonomic Dysfunction and Increases Locomotor Activity in Sgcd^{-/-} Mice without Lowering Blood Pressure

Mean arterial pressure (MAP, 24 hr avg) was significantly lower in Sgcd^{-/-} mice compared with control mice, whereas mean HR did not differ between the genotypes (Fig 1A, 1B). Sgcd^{-/-} mice exhibited significant decreases in spontaneous BRS (Fig 1C) and vagal tone (Fig 1D), and an increase in sympathetic vasomotor tone (Fig 1F). The increase in cardiac sympathetic tone did not reach statistical significance (Fig 1E). Locomotor activity was markedly decreased in Sgcd^{-/-} mice (Fig 1G). We have reported similar findings in our earlier studies of young Sgcd^{-/-} mice (12, 13).

Oral administration of CD-Ang-(1-7) for 8–9 wks reduced HR, increased BRS and cardiac vagal tone, and decreased cardiac and vasomotor sympathetic tone without further lowering MAP in Sgcd^{-/-} mice (Fig 1A-1F). CD-Ang-(1-7) also increased locomotor activity in Sgcd^{-/-} mice (Fig 1G). Each of these measurements in treated Sgcd^{-/-} mice were restored to levels not different than those measured in control mice (Figs 1B-1G). The beneficial effects of CD-Ang- (1-7) were selective in that the treatment had essentially no effect on these variables in control mice (Figs 1A-1G).

CD-Ang-(1-7) Decreases Fibrosis and Attenuates Oxidative Stress in Skeletal Muscle of Sgcd^{-/-} Mice

Skeletal muscle fibrosis and oxidative stress are hallmark pathological features in muscular dystrophy [16, 21, 22, 23]. As expected, total fibrosis, collagen III, fibronectin and superoxide levels were elevated in skeletal muscle of Sgcd^{-/-} mice (Figs 2A-2C). Treatment with CD-Ang- (1-7) markedly reduced fibrosis and superoxide in Sgcd^{-/-} mice

to levels near those observed in control mice (Figs 2A-2C). CD-Ang-(1-7) treatment had essentially no effect on fibrosis or superoxide in control mice (Figs 2A-2C).

CD-Ang-(1-7) Alters AT₁R and Mas Protein Expression in Dystrophic Skeletal Muscle

Ang II binding to AT₁R generates superoxide and upregulates AT₁R expression in states of chronic RAS activation [24, 25]. As we predicted, AT₁R expression measured by Western blot and immunofluorescence was significantly increased in quadriceps muscle of Sgcd^{-/-} mice, while Mas expression was significantly reduced (Figs 3, 4). Treatment of Sgcd^{-/-} mice with CD-Ang-(1-7) abolished these differences (Figs 3, 4). CD-Ang-(1-7) did not alter the expression of AT₁R or Mas in control mice (Fig 3, 4). AT₁R immunofluorescence in skeletal muscle averaged 15 ± 3 , 13 ± 2 , 25 ± 4 , and 15 ± 3 units in untreated C57BL/6, treated C57BL/6, untreated Sgcd^{-/-}, and treated Sgcd^{-/-} mice, respectively. Conversely, Mas immunofluorescence averaged 10 ± 4 , 14 ± 5 , 3 ± 1 , and 14 ± 3 units in these same groups of mice.

DISCUSSION

Muscular dystrophies are a group of heterogeneous genetic disorders that cause progressive muscle weakness and wasting, cardiac and respiratory abnormalities, loss of mobility and early mortality. Although each of the individual muscular dystrophies is relatively rare, they collectively affect millions of people worldwide and share a synergistic, pathological pathway of myofiber degeneration and atrophy. There is no cure for muscular dystrophy and current therapies are limited.

The major findings of the present study are that treatment of Sgcd^{-/-} mice with CD-Ang-(1-7): (1) Markedly reduces fibrosis and oxidative stress in skeletal muscle accompanied by restoration of the balance between AT₁R and Mas expression; (2) Reverses autonomic dysregulation, i.e., increases cardiac vagal tone and BRS, and decreases cardiac and vasomotor sympathetic tone; and (3) Restores spontaneous locomotor activity to levels similar to that measured in control mice. The results suggest that these beneficial effects of Ang-(1-7) are mediated at least in part by inhibition of the Ang II/AT₁R axis.

The importance of activation of skeletal muscle RAS and autonomic dysregulation in the pathogenesis of multiple types of muscular dystrophy is now widely recognized [4, 5, 14–16, 27–28]. We recently demonstrated that Sgcd^{-/-} mice exhibit skeletal muscle RAS activation and severe autonomic dysregulation at a young age before onset of cardiac dysfunction [12, 13], results that were confirmed in the present study. In addition, we showed that levels of Ang II are increased in both skeletal muscle and plasma in young Sgcd^{-/-} mice [13]. The increased circulating levels of Ang II may contribute to the autonomic dysregulation. Ang II exerts actions at multiple central and peripheral nervous system sites that facilitate an increase in sympathetic tone and decreases in vagal tone and BRS [18, 30–32].

In addition, oxidative stress, acidic pH and/or inflammation in dystrophic muscle may cause autonomic dysregulation by activating sensory nerves in muscle and enhancing sympathoexcitatory reflexes [33–36]. AT₁Rs are expressed in sensory nerve terminals in

muscle [37, 38]. Furthermore, AT₁Rs may function as mechanosensors and contribute to activation of mechanosensitive nerve terminals [39]. Ang II/AT₁R promotes fibrosis by increasing extracellular proteins collagen III and fibronectin, oxidative stress and TGF β expression in the skeletal muscles [25].

Deleterious effects of activation of Ang II/AT₁R are countered by protective effects mediated by the Ang-(1-7)/Mas axis in a variety of pathological states [7, 8, 31,40–43]. We found that oral administration of Ang-(1-7) encapsulated in hydroxypropyl β -cyclodextrin to Sgcd $^{-/-}$ mice for 8–9 wks significantly reduced fibrosis and oxidative stress in skeletal muscle, improved autonomic regulation (increased BRS and cardiac vagal tone, decreased sympathetic tone and HR), and normalized locomotor activity. Importantly, these beneficial effects of Ang-(1-7) were achieved through oral administration and occurred at a dose that did not lower MAP. The use of β - cyclodextrin to facilitate absorption and the half-life of Ang-(1-7) is of key importance to the effectiveness of the compound administered orally [17], and increases the potential to translate this therapy to patients.

As Ang-(1-7) was administered systemically *in vivo*, the cellular and molecular targets responsible for mediating its beneficial effects on skeletal muscle, autonomic regulation and locomotor activity in Sgcd $^{-/-}$ mice remain to be determined. Ang-(1-7) has been shown to exert protective effects in numerous organs/tissues including blood vessels, heart, brain, lungs, liver, kidneys, adipose tissue and skeletal muscle [7, 8, 40–42]. In an elegant study published recently, Acuna et al. showed that CD-Ang-(1-7) restored muscle strength and reduced fibrosis by inhibiting TGF- β signaling in the gastronemius, tibialis anterior, and diaphragm skeletal muscles of *mdx* mice, a model of Duchenne muscular dystrophy [44]. Ang-(1-7) has also been shown to improve insulin sensitivity in skeletal muscle of fructose-fed rats, a model of metabolic syndrome, and to attenuate Ang II-induced inhibition of insulin signaling in a Mas receptor-dependent manner, both *in vivo* and *in vitro* [45, 46]. Chronic treatment with Ang-(1-7) has been reported to decrease plasma renin activity and plasma aldosterone concentration in fructose-fed rats [47]. Such an effect could contribute to changes in autonomic regulation and control of body fluid balance. We suspect that Ang-(1-7) may exert protective actions at multiple sites including those within skeletal muscle and the brain, reflecting the numerous, documented sites of actions of Ang II and Ang-(1-7) that mediate their effects on skeletal muscle, blood vessels, heart and the autonomic nervous system [8, 11, 41, 42, 47–50]. Future studies are needed to determine the key sites of action of Ang-(1-7) in mediating its protective effects in muscular dystrophy.

A chronic peptide imbalance in which the Ang II/AT₁R axis is increased and the Ang-(1-7)/Mas axis is decreased is evident in aging, hypertension, and other pathological states [32, 43]. These findings predict that a balance between Ang II/AT₁R and Ang-(1-7)/Mas is essential for normal organ system function. The decreased expression of Mas and increased expression of AT₁R in skeletal muscle of untreated Sgcd $^{-/-}$ mice along with restoration of the peptide balance after treatment with Ang-(1-7) support this concept.

Previously, we reported that the degree of autonomic dysfunction in Sgcd $^{-/-}$ mice at a young age predicts the severity of cardiac dysfunction and mortality at a later age [12]. We now speculate that improving autonomic and skeletal muscle function at a young age by

treatment of *Sgcd*^{-/-} mice with Ang-(1-7) will delay the onset of DCM and prolong lifespan.

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ABBREVIATIONS FOOTNOTE

LGMD2F	Limb girdle muscular dystrophy-2F
Sgcd	sarcoglycan delta
Ang II	angiotensin II
AT₁R	angiotensin II type 1 receptor
AT₂R	angiotensin II type 2 receptor
Ang-(1-7)	angiotensin-(1-7)
Mas	Mas receptor
ACE2	angiotensin converting enzyme 2
ACE	angiotensin converting enzyme
TGFβ	transforming growth factor beta
BP	blood pressure
MAP	mean arterial pressure
HR	heart rate
BRS	baroreflex sensitivity
HRP	horseradish peroxidase
DAB	3,3'-diaminobenzidine
O₂^{•-}	superoxide
DAPI	4',6-diamidino-2-phenylindole
DHE	dihydroethidium

CLINICAL PERSPECTIVES

Muscular dystrophy is a catastrophic disease that is in need of new therapies to delay pathogenicity of disease and improve quality of life in the afflicted population. In the present study, we show that *Sgcd*^{-/-} mice (model of LGMD2F) exhibit severe autonomic and skeletal muscle dysfunction at a young age. Chronic administration of Ang-(1-7) in *Sgcd*^{-/-} mice (before onset of cardiac dysfunction) restores autonomic function, increases locomotor activity, and attenuates skeletal muscle fibrosis and oxidative stress. These protective benefits arise from restoration of balance between the increased Ang II/AT₁R and reduced Ang-(1-7)/Mas axes. Thus, our results provide strong support for the use of Ang-(1-7) or enhancement of its endogenous production as a novel therapeutic approach to correct the early autonomic dysfunction and delay the pathological consequences in muscular dystrophy.

SUMMARY STATEMENT

Muscular dystrophy is a devastating disease that causes muscular weakness, heart problems and early mortality. Currently, there is no cure for it. Our study provides strong evidence for a novel therapeutic target that can delay pathogenesis of this disease.

Figure 1. Blood pressure, HR autonomic indices and locomotor activity in untreated and CD-Ang-(1-7) treated control and Sgcd-/- mice

Sgcd-/- mice exhibited lower mean blood pressure and locomotor activity, and severe autonomic dysfunction at a young age compared with control mice. CD-Ang-(1-7) prevented the autonomic dysfunction, reduced heart rate without further lowering blood pressure, and markedly improved locomotor activity in Sgcd-/- mice. Results from untreated control (n=5, black bars), treated control (n=6, gray bars), untreated Sgcd-/- (n=7, black bars), and treated Sgcd-/- (n=8, gray bars) mice are shown. *P < 0.05, Sgcd-/- vs.

C57BL/6 mice; $\dagger P < 0.05$, vs. untreated mice (unpaired t-test). **Panel A.** 24 hr average of mean blood pressure. **Panel B.** 24 hr average of heart rate. **Panel C.** Baroreflex Sensitivity. **Panel D.** Cardiac vagal tone measured as change in heart rate in response to methylatropine. **Panel E.** Cardiac sympathetic tone measured as a change in heart rate in response to propranolol. **Panel F.** Vasomotor sympathetic tone measured as a change in blood pressure in response to chlorisondamine. **Panel G.** 24 hr average of spontaneous locomotor activity.

Figure 2. Fibrosis, fibronectin, collagen III, and superoxide ($O_2^{\bullet-}$) in skeletal muscle of untreated and Ang-(1-7)-treated control and Sgcd-/- mice

Panel A. Left: Fibrosis indicated by blue staining (Masson Trichrome) in tissue sections of quadriceps muscle from control and Sgcd-/- mice (magnification 20X; scale bar 50 microns). Right: Fibrosis is expressed as a percentage of the total area examined in quadriceps from untreated control (n=6), treated control (n=5), untreated Sgcd-/- (n=9), and treated Sgcd-/- (n=5) mice. **Panel B.** Fibronectin and collagen III indicated by dark brown color (HRP/DAB kit) in quadriceps skeletal muscle from control and Sgcd-/- mice

(magnification 20X; scale bar 50 microns). A negative control is shown for tissue of Sgcd^{-/-} mouse exposed to secondary antibody (IgG). Similar results of fibronectin and collagen III staining were obtained from other untreated control (n=4), treated control (n=4), untreated Sgcd^{-/-} (n=5), and treated Sgcd^{-/-} (n=4) mice. **Panel C.** Left: O₂^{•-} indicated by dihydroethidium (DHE) fluorescence (red) in 7µm sections of quadriceps muscle from control and Sgcd^{-/-} mice (magnification, 20X; scale bar, 50 microns). Right: Quantification of O₂^{•-} in muscle from untreated control (n=5), treated control (n=4), untreated Sgcd^{-/-} (n=4), and treated Sgcd^{-/-} (n=4) mice. Fluorescence is expressed as a percentage of the mean level of fluorescence measured in muscle from untreated control mice. CD-Ang-(1-7) markedly attenuated fibrosis, fibronectin, collagen III and O₂^{•-} levels in Sgcd^{-/-} mice, with no effect on either measurement in control mice. Data in this figure are from untreated (black bars), and CD-Ang-(1-7)-treated (gray bars) groups. *P < 0.05, Sgcd^{-/-} vs. control mice; †P < 0.05, vs. untreated mice (unpaired t-test).

A. AT₁R in Skeletal Muscle

B. MasR in Skeletal Muscle

Figure 3. Expression of AT₁R and Mas receptors in skeletal muscle of untreated and CD-Ang-(1-7)-treated control and Sgcd^{-/-} mice

Panel A. Immunofluorescence showing increased AT₁R-immunoreactivity in quadriceps muscle of untreated Sgcd^{-/-} vs. control mice, but normal expression in CD-Ang-(1-7)-treated Sgcd^{-/-} mice. Confocal image of muscle with AT₁R staining in green (magnification, 20X; scale bar, 100 microns). **Panel B.** Immunofluorescence showing reduced Mas-immunoreactivity in quadriceps muscle of untreated Sgcd^{-/-} vs. control mice, but normal expression in Ang-(1-7)-treated Sgcd^{-/-} mice. Confocal images of muscle with

Mas staining in green and nuclei staining in blue (magnification, 20X; scale bar, 100 microns). A negative control is shown for quadriceps muscle exposed to secondary antibody (IgG) and nuclear stain DAPI (4',6-diamidino-2-phenylindole) in control and *Sgcd*^{-/-} mice.

Western Blot in Skeletal Muscle

Figure 4. Western Blot Analysis of AT₁R and Mas receptors in skeletal muscle of untreated and CD-Ang-(1-7)-treated control and Sgcd^{-/-} mice

Western blots showing increased AT₁R and reduced Mas protein expression in skeletal muscle (quadriceps) of untreated Sgcd^{-/-} vs. control mice. CD-Ang-(1-7) treatment increased Mas and reduced AT₁R expression in Sgcd^{-/-} mice. Bottom: Group data obtained from untreated control (n=4, black bar), treated control (n=6, gray), untreated Sgcd^{-/-} (n=4, black) and treated Sgcd^{-/-} (n=6, gray) mice. *P < 0.05, Sgcd^{-/-} vs. control mice; †P < 0.05, vs. untreated mice (unpaired t-test).