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IGF-1 Prevents Diastolic and Systolic Dysfunction Associated with Cardiomyopathy and Preserves Adrenergic Sensitivity

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Abstract

Aims—Insulin-like growth factor 1 (IGF-1)-dependent signaling promotes exercise-induced physiological cardiac hypertrophy. However, the in vivo therapeutic potential of IGF-1 for heart disease is not well established. Here we test the potential therapeutic benefits of IGF-1 on cardiac function using an in vivo model of chronic catecholamine-induced cardiomyopathy.

Methods—Rats were perfused with isoproterenol via osmotic pump (1 mg/kg/day) and treated with 2 mg/kg IGF-1 (2 mg/kg/day, 6 days a week) for 2 or 4 weeks. Echocardiography, ECG, and blood pressure were assessed. In vivo pressure-volume loop studies were conducted at 4 weeks. Heart sections were analyzed for fibrosis and apoptosis, and relevant biochemical signaling cascades were assessed.

Results—After 4 weeks, diastolic function (EDPVR, EDP, tau, E/A ratio), systolic function (PRSW, ESPVR, dP/dtmax), and structural remodeling (LV chamber diameter, wall thickness) were all adversely affected in isoproterenol-treated rats. All these detrimental effects were attenuated in rats treated with Iso+IGF-1. Isoproterenol-dependent effects on BP were attenuated by IGF-1 treatment. Adrenergic sensitivity was blunted in isoproterenol-treated rats but was preserved by IGF-1 treatment. Immunoblots indicate that cardioprotective p110 α signaling and activated Akt are selectively upregulated in Iso+IGF-1 treated hearts. Expression of iNOS was

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Conflict of interest
None declared.

significantly increased in both the Iso and Iso+IGF-1 groups, however tetrahydrobiopterin (BH4) levels were decreased in the Iso group and maintained by IGF-1 treatment.

Conclusion—IGF-1 treatment attenuates diastolic and systolic dysfunction associated with chronic catecholamine-induced cardiomyopathy while preserving adrenergic sensitivity and promoting BH4 production. These data support the potential use of IGF-1 therapy for clinical applications for cardiomyopathies.

Keywords

IGF-1; adrenergic stimulation; cardiomyopathy; tetrahydrobiopterin; diastolic dysfunction; systolic dysfunction

Introduction

Heart failure (HF) affects nearly six million people in the United States results in ~350,000 deaths annually (Roger et al., 2012). HF is typically associated with impaired systolic function. However, approximately half of the deaths associated with HF result from complications stemming from diastolic dysfunction, or the inability of the heart to relax (Halley et al., 2011). While treatment options for systolic dysfunction, like beta blockers and ACE inhibitors, have been mainstays for over 30 years, treating patients suffering from diastolic dysfunction is severely limited. No drug, device, or therapy is indicated for the treatment of diastolic heart failure. Current medical strategies primarily address the associated underlying pathologies (i.e. hypertension, diabetes, obesity) and fail to target cardiac function. A new approach to treating HF is needed.

Insulin-like growth factor 1 (IGF-1) is the anabolic effector of growth hormone production and promotes exercise-induced, physiological cardiac hypertrophy. IGF-1 increases contractility, induces angiogenesis after myocardial infarct, and delays myocyte apoptosis (Chen et al., 2000, Cittadini et al., 1998, von Lewinski et al., 2003). Interestingly, several studies have linked IGF-1 deficiency with HF, and lower serum IGF-1 levels are independently associated with all-cause mortality (Roubenoff et al., 2003, Fazio et al., 1996). Notably, over the last decade, exercise training has emerged as an adjunct therapy for the cardiac dysfunction associated with HF (Downing and Balady, 2011). The precise mechanism(s) by which exercise therapy mediates the clinical improvement of HF patients is not fully understood. These observations have led to an increased interest in the use of IGF-1 as a potential therapeutic against HF.

During the progression of heart failure, cardiac function deteriorates and cardiac output is compromised. In response to this loss of function, the cardiovascular system relies on increased adrenergic tone to maintain proper blood perfusion. While initially a compensatory mechanism, chronic adrenergic signaling becomes maladaptive with time. When prolonged it leads to apoptosis of cardiomyocytes, further weakening the heart. Chronic adrenergic tone also leads to blunted contractility, electrical remodeling, arrhythmogenesis, and pathological hypertrophy (Engelhardt et al., 2004). As such, chronic adrenergic signaling plays a key role in the development of HF. Similarly, excess IGF-1 signaling is also detrimental such as found in patients with acromegaly. These patients

present with enlargement of body organs (including cardiomegaly) and often suffer from severe congestive HF at early ages (Palmeiro et al., 2012). Therefore, when unchecked these separate signaling cascades adversely affect human cardiac physiology.

However, the physiological context in which these signals exist may determine how the heart responds to them. Intriguingly, in the heart of the trained athlete, chronic adrenergic tone and persistent IGF-1 signaling coexist (Neri Serneri et al., 2001, Barbier et al., 2006). Yet, the underlying hypertrophy in the athletically trained heart is quite dissimilar from that found in patients suffering from heart failure. This suggests that the expected hypertrophic fate induced by chronic adrenergic stimulation is somehow being modulated or altogether changed in these subjects. In the broadest terms, athletic hypertrophy is associated with enhanced cardiac function with no increases in mortality. On the contrary, pathological hypertrophy is associated with decreased cardiac function and increased mortality (Weeks and McMullen, 2011). From this we speculate that it is the combination of adrenergic- and IGF-1-dependent signaling that when present together mediate physiological hypertrophy. The ability of IGF-1 to modify maladaptive adrenergic signaling has never been directly tested.

We hypothesized that IGF-1 stimulation would improve the cardiac dysfunction observed in the setting of chronic catecholamine-induced cardiomyopathy. To test this, we subjected rats to chronic infusion of isoproterenol (Iso) $\pm$ IGF-1 (2 mg/kg/day, 6 days a week) via osmotic pump for 2 or 4 weeks and measured in vivo cardiac function. Our findings demonstrate that IGF-1 treatment positively impacts both diastolic and systolic dysfunction and attenuates adrenergic desensitization associated with chronic catecholamine-induced cardiomyopathy. The activation of certain phosphoinositide 3-kinases (PI3K) are established mediators of both physiological and pathological cardiac hypertrophy (Dorn and Force, 2005). Mechanistically, we find the expression of two class I PI 3-kinase subtypes, p110 α and p110 γ , to be increased in hearts treated with Iso + IGF1 along with increased Akt phosphorylation. Expression of inducible nitric oxide synthase (iNOS) was 3—4-fold higher in rats treated with Iso alone or Iso+IGF-1. However, treatment with Iso+IGF-1 rescued the expression of the iNOS cofactor, tetrahydrobiopterin (BH4), indicating that IGF-1 promotes the coupling of iNOS to nitric oxide production. This is the first report to indicate that IGF-1 regulates BH4 production in the heart. Our data show that in the setting of chronic sympathetic drive, IGF-1 treatment shifts the hypertrophic remodeling from a maladaptive response state toward a more physiological, or athletic-like, state.

Materials and Methods

Experiments were reviewed and approved by OSU and QTest Labs Institutional Animal Care and Use Committee (IACUC) and conformed to the Guide for the Care and Use of Laboratory Animals published by the NIH (publication No. 85-23, revised 1985). This study conforms with Good Publication Practice in Physiology 2013 Guidelines for *Acta Physiologica*. *Acta Physiol (Oxf)*. (Persson, 2013)

Experimental model

Adult male Sprague-Dawley rats, 10–12 weeks old, approximately 300 g, were used in this study. To induce hypertrophy, either isoproterenol (Iso, Sigma, St. Louis, MO), recombinant human insulin growth factor-1 (IGF-1, Increlex [mecasermin], Ipsen, Paris, France), or the combination of both were used. Iso was chronically perfused using Alzet mini-osmotic pumps (Alzet, Cupertino, CA). This model well characterized and has been demonstrated to induced irreversible hypertrophy by 7 days of Iso infusion (Takeshita et al., 2008). Animals were anesthetized by intraperitoneal (IP) ketamine/xylazine (~45/~5mg/kg). An incision (~1 cm) was made for Alzet model #2002 (2 weeks) or #2004 (4 weeks), and pumps were implanted according to the manufacturer's instructions. For Iso and Iso+IGF-1 groups, pumps were filled with Iso and chronically perfused at 1 mg/kg/day. Pumps for control and IGF-1 groups were filled with 0.9% NaCl normal saline. IGF-1 (2 mg/kg/day) was administered SQ (6 days/week, starting on the day on implantation). Control and Iso rats were injected with a similar volume of saline.

Echocardiography

One hour prior to echocardiography measurements, rats were anesthetized by IP injection of ketamine/xylazine (~45/~5mg/kg) and the Iso or saline pump was harvested. A half dose of ketamine/xylazine (~22.5/~2.5mg/kg) was administered IP immediately prior to measuring left ventricular function and geometry (Phillips HD11xe). Transthoracic M-mode examinations at the mid-papillary region were performed to assess septal wall thickness, chamber size, ejection fraction, and fractional shortening. Doppler echo was used to assess the ratio of the early (E) to late (A) ventricular filling velocities (E/A ratio). At the time of echocardiography the heart rates for all treatments were not significantly different (Control = 222 ± 13 ; Iso = 233 ± 24 ; Iso + IGF1 = 249 ± 33 ; Iso + IGF1 = 243 ± 11 , n.s.).

Pressure-Volume Loop Studies and ECG

Following echo measurements, rats were anesthetized with sodium pentobarbital (IP, 45 mg/kg), intubated, and ventilated. Anesthesia was maintained with continuous sodium pentobarbital IV infusion (4 mg/kg/hr) until completion of the experiment. Transthoracic needle electrodes forming a single-lead ECG (lead II) were placed. For LV mechano-energetic evaluation, a 2F high-fidelity conductance/micromanometer catheter (Millar Instruments) was advanced retrograde across the aortic valve and into the LV chamber to simultaneously determine left-ventricular pressure and volume. A separate 2F high-fidelity micromanometer catheter (Millar Instruments) was inserted into a femoral artery and advanced towards the abdominal aorta to record arterial pressures. An appropriately sized balloon catheter was placed and advanced into the inferior vena cava via left femoral vein. When inflated acutely, decreased myocardial preload enabled the generation of a family of pressure-volume (PV) curves/loops. The resulting left-ventricular PV data was analyzed offline using dedicated software (IOX/ECG Auto; EMKA Technologies). Dobutamine challenge was performed by administering escalating doses of 1, 2.5, 5, and 10 $\mu\text{g/kg/min}$. The carotid-femoral estimated pulse wave velocity was measured as the difference between the onset of the arterial pressure in the carotid and in the femoral artery.

Histopathology

Hearts were sectioned (4 μ m) and fixed in 10% neutral buffered formalin and processed for routine histopathology. For histopathology analysis, sections were paraffin embedded to allow visualization of all four cardiac chambers. Sections were stained with hematoxylin and eosin and Masson's trichrome. An independent pathology core assessed tissue sections for evidence of fibrosis. Briefly, a qualitative score was initially given to each section on a scale of 1–5: 1, no fibrosis; 2 slight fibrosis; 3, moderate fibrosis; 4, heavy fibrosis; 5, severe fibrosis. Scores were: control, 1.33 ± 0.21 ; IGF alone 1.16 ± 0.17 ; Iso, 4.0 ± 0.37 ; Iso+IGF1 2.8 ± 0.58 . These scores agreed with the quantitative findings. Quantitative assessment of fibrosis was conducted on the ImageJ software (Schneider et al., 2012) (NIH, Bethesda, MD) using the threshold analysis previously described (Hadi et al., 2011), and adapted for Masson's trichrome. Prior to analysis, tissue sections were digitally imaged at 3,000 dpi using the PathScan Enabler IV (Meyer Instruments, Houston, TX). Immunohistochemistry for cleaved caspase-3 was performed under the following conditions: antigen retrieval conducted with Target Retrieval Solution (Dako, Carpinteria, CA) pH 6.0 citrate buffer; rabbit polyclonal anti-caspase-3 (Cell Signaling Technology) was applied at 1:100 for 30 minutes; sections were washed and biotinylated goat anti-rabbit IgG was applied at 1:500 (Vector BA1000, Burlingame, CA); chromagen was DAB (Dako). Sections were screened for the presence of cleaved caspase-3 under a 60x objective. Twenty separate high power fields (HPF) were examined for each section. Data are reported as the average number of positively stained cells per HPF. Rat spleen served as positive control.

Immunoblot studies

Immunoblots were conducted as previously described (Gudmundsson et al., 2012). Briefly, tissue lysates were analyzed on Mini-PROTEAN tetra cell (BioRad) on a 4–15% precast TGX gel (BioRad). Gels were transferred to a nitrocellulose membrane using the Mini-PROTEAN tetra cell (BioRad). Densitometry analysis was done using ImageLab software (BioRad). For all experiments, protein values were normalized against an internal loading control (GAPDH, Sigma). Polyclonal rabbit antibodies against p110 α (1:1000), p110 γ (1:1000), (EMD Millipore, Billerica, MA), Akt (1:2000, Cell Signaling Technology, Boston, MA), phospho-Akt-Ser473 (1:2000, Cell Signaling Technology), iNOS (1:1000, Cell Signaling) were applied to nitrocellulose membrane in 5% milk, T-TBS buffer, overnight at 4° C.

Fluorescence High Performance Liquid Chromatography Detection of Pteridines

The HPLC analysis of pteridines BH4 and BH2 was performed as previously reported using fluorescence detection with excitation set at 348 nm and emission set at 444 nm (Fukushima and Nixon, 1980, De Pascali et al., 2014). This method was chosen for its sensitivity and selectivity in the detection of BH4 and BH2 after controlled oxidation to pterin and biopterin, respectively. Hearts were ground in liquid nitrogen and dounce homogenized in ice cold 0.1 N HCl degassed by argon. Resulting homogenate was added 1:1 with a 2% KI/3% iodine, .2 N KOH solution and allowed to react for 1 hour at ambient temperature in the dark. Reaction mixtures were then centrifuged at 20,000 x g for 10 minutes and the resulting supernatant was filtered through a 3000 MWCO regenerated cellulose filter for

direct HPLC injection. The HPLC analysis of pteridines was carried out using a Waters Atlantis T3 reversed phase column (4.6×150 mm). Isocratic elution of pteridines was performed at a flow rate of 1.2 mL/min using a buffer consisting of 100 mM KH_2PO_4 , 6 mM citric acid, 2.5 mM sodium octyl sulfate and 2% methanol, pH 2.5. Peaks were assigned by co-elution with analytical standards, and quantitation was performed with use of standard curves prepared from analytical standards.

Statistics—All values are presented as mean $\pm$ SEM. Data were analyzed by one way ANOVA or two way ANOVA, when appropriate, with a Bonferroni post-test applied when statistical significance was indicated. All statistical analysis was conducted in GraphPad Prism V4.01. P-values <0.05 were considered significant.

Results

IGF1 attenuates structural remodeling and improves hemodynamics

Echocardiographic cardiac structural parameters are summarized for control and experimental rats in Table 1. IVS_s , IVS_d , LVID_d , and LVID_s were all significantly increased after 4 weeks of treatment by Iso alone compared to control, while only the IVS_s and IVS_d were increased in Iso+IGF-1 group. Ejection fraction (EF) was also significantly lower in the Iso treated group at 2 and 4 weeks compared to both control and Iso+IGF-1. HW/BW was significantly increased by Iso and Iso+IGF-1 treatment at both endpoints. These data indicate that Iso treatment had the expected effects on hypertrophy, chamber dilation, and ventricular contractility. Treatment with Iso+IGF-1 had a similar effect on septal wall thickness and HW/BW. However, rats treated with Iso+IGF-1 did not exhibit the severity of dilation or depressed ejection fraction observed in the Iso group. This indicates that the hypertrophic fate present in the Iso+IGF-1 group is divergent from that of the Iso group, and that this fate is more athletic-like.

IGF-1 is suggested to protect against cardiac fibrosis and apoptosis (Boucher et al., 2008, Huynh et al., 2010). We analyzed whole heart tissue sections for evidence of fibrosis or myocyte apoptosis using Masson's trichrome staining and cleaved caspase-3 immunohistochemistry, respectively. We observed fibrosis after 2 and 4 weeks of treatment in the Iso group, especially within the endo- to mid-myocardium of the apex (Figures 1A–C). While fibrosis was visually increased in the Iso+IGF-1 treated group, this increase failed to reach statistical significance (Figure 1C). Neither the control nor the IGF-1 alone group developed fibrosis by 4 weeks, indicating that fibrosis development was likely Iso-dependent. Quantitative analysis of cleaved caspase-3 revealed no differences comparing all treatments (Figure 1C). While the observed increase in fibrosis suggests increased apoptosis, the caspase-3 assay may be insensitive to the changes in apoptosis at the 4 week time point.

Adrenergic stimulation is well known to increase vascular resistance. As expected, we observed a significant increase in mean arterial blood pressure (MAP) in Iso-treated rats compared to control also, 115.1 ± 5.7 , Control, 85.5 ± 4.3 , mmHg, $p < 0.01$, Figure 1D). This Iso-dependent effect on MAP was completely attenuated by treatment if IGF-1 (Iso+IGF-1, 88.7 ± 4.7 , 81.9 ± 3.9 mmHg). As a measure of vascular compliance, we assessed pulse wave velocity; an increased velocity indicates a less compliant vasculature. We found pulse wave

velocity to be significantly faster in Iso (20.0 ± 4.3 m/s) compared to control (8.1 ± 1.2 m/s, $p < 0.01$, Figure 1E). No differences were observed in the Iso+IGF-1 group (7.8 ± 0.9 m/s). Together these data suggest that IGF-1 treatment decreases BP likely through an effect on arterial compliance and may, in part, explain the attenuated structural remodeling in the Iso+IGF-1 treated hearts.

Diastolic dysfunction in chronic catecholamine-treated rats is attenuated by IGF-1 treatment

Chronic catecholamine stimulation is linked with the development of diastolic dysfunction. Therefore, we assessed the ability of IGF-1 treatment to attenuate diastolic dysfunction associated with chronic Iso treatment. *In vivo* cardiac function was assessed by PV loop studies after 4 weeks of treatment. It is important to note that many derived PV loop parameters are independent of preload and afterload. Thus, we are able to isolate the direct effect of IGF-1 on myocardial performance independent of co-variables such as blood pressure. We assessed the end diastolic PV relationship (EDPVR), a direct index of myocardial compliance. Figure 2A shows a representative family of curves and their relative slopes of the EDPVR for these individual traces. The average EDPVR slope in all treatments is showing in Figure 2B. The EDPVR was significantly higher in Iso-treated rats compared to control Iso+IGF-1 groups (2.55 ± 0.28 , 1.08 ± 0.18 , $p < 0.01$, Figures 2A & 2B), indicating diastolic dysfunction in this group. This Iso-dependent effect on ventricular compliance was relieved in the Iso+IGF-1 group (1.42 ± 0.12).

Prolonged QT interval independently associates with diastolic dysfunction in human heart failure (Wilcox et al., 2011). Using ECG, we demonstrate that the rate corrected QT (QT_{CB}) was significantly increased by chronic Iso treatment compared to control (215.2 ± 15.9 vs. 168.0 ± 12.9 ms, $p < 0.05$, Figure 2C). This prolonged QT_{CB} was not observed in the Iso+IGF-1 group (167.5 ± 12.0 ms), similar to what is observed in exercise-induced hypertrophy.

By echocardiography, we determined the E/A ratio to be significantly shifted in favor of late ventricular filling in Iso-treated rats (2.02 ± 0.08 vs. 1.35 ± 0.06 , control vs. Iso, respectively, $p < 0.001$, Figure 2D). This data indicates an increased reliance on the atrial contraction to reliably fill the ventricle in Iso-treated rats, an observation consistent with diastolic dysfunction observed in the clinic. Treatment with IGF-1 significantly attenuated this shift in the E/A ratio (1.71 ± 0.12 , $p < 0.05$ vs. Iso alone, N.S. vs. control).

Increases in end diastolic pressure (EDP) are indicative of the development of congestive failure and diastolic dysfunction. Therefore, we assessed EDP both at rest and in response to the adrenergic agonist, dobutamine. At baseline the Iso treated rats had significantly higher EDP compared to all other treatments (Figure 2E). Similarly, EDP remained elevated in the Iso treated rats after dobutamine stimulation. These Iso-dependent effects were attenuated by treatment with IGF-1 (Figure 2F). The tau of relaxation is a direct measure of myocardial relaxation independent of preload and afterload. The tau was significantly longer in Iso-treated rats compared to control and Iso+IGF-1-treated rats (13.25 ± 0.44 , 9.05 ± 0.42 , 8.99 ± 0.44 ms, respectively, $p < 0.0001$, Figure 2G). In response to dobutamine, the tau of relaxation remained significantly slower in Iso-treated hearts but was normalized in the Iso

+IGF-1 group. In line with this, the minimum rate of pressure change (dp/dt_{min} , Figure 2H) was also decreased by Iso treatment and attenuated by treatment with IGF-1. Together, these data demonstrate that the diastolic dysfunction associated with chronic β -AR stimulation is mitigated by simultaneous treatment with IGF-1.

Systolic function and adrenergic response is preserved in chronic catecholamine-treated rats by treatment with IGF-1

Chronic adrenergic stimulation is well known to blunt myocardial contractility. Indeed, we observed this loss of contractility in Iso-treated rats (Figure 3). As IGF-1 is known to positively affect myocardial inotropy (von Lewinski et al., 2003), we assessed the effect of IGF-1 treatment on systolic function. In our PVL studies, the slope of the end systolic pressure-volume relationship (ESPVR) was decreased by Iso treatment, indicating decreased contractility (Figure 2A & 2B). This relationship was preserved by treatment with IGF-1 (Figure 3A & 3B). The slope of preload recruitable stroke work (PRSW), the gold standard and direct measure of contractility, was significantly lower in rats treated with Iso compared to control (34.5 ± 1.3 , 53.4 ± 0.88 , respectively, $p < 0.0001$, Figure 3C). PRSW was partially rescued by treatment with IGF-1 (46.4 ± 1.5 , $p < 0.0001$ vs. Iso alone), but remained lower than observed in control ($p < 0.05$ vs. control). These differences were maintained following dobutamine challenge, indicating a preservation of adrenergic responsiveness in the Iso + IGF-1 rats despite chronic catecholamine stimulation. Adrenergic responsiveness was also measured by cardiac output, heart rate, and dp/dt_{max} in a dose response to dobutamine (Figures 3D–F). In all these measures, Iso treated rats had a significantly blunted response to adrenergic stimulation, and this response was rescued in the Iso+IGF-1 group (Figures 3D–F). As cardiac output also reflects stroke work and diastolic filling, this data highlights the preserved diastolic function and structural changes observed in the Iso+IGF-1 group. Lastly, in line with the blood pressure data in Figure 1, as measured by PV loops, arterial elastance was significantly increased in the Iso treated rats, and this was normalized by IGF-1 treatment (Figure 3G), indicating that the Iso-dependent increase in vascular resistance was normalized by IGF-1 treatment. Together, these data indicate that IGF-1 treatment partially preserves systolic function. Importantly, these data demonstrate that despite persistent catecholamine stimulation and the expected loss of adrenergic sensitivity, rats treated with Iso+IGF-1 maintain adrenergic responsiveness. Our data are the first to show that IGF-1- and β -AR-dependent signals crosstalk.

IGF-1 treatment promotes the activation of cardioprotective signaling cascades

Activation of p110 α and p110 γ are believed to mediate physiological and maladaptive cardiac hypertrophy, respectively. Mice deficient of p110 α are unable to remodel in response to exercise training, while mice deficient in p110 γ or expressing a dominant negative p110 γ are protected against maladaptive hypertrophy (McMullen et al., 2003, Oudit et al., 2003, Nienaber et al., 2003, Patrucco et al., 2004). However, the simultaneous activation of p110 α signaling (through IGF-1) and p110 γ signaling (through catecholamines) on hypertrophy has never been tested. We hypothesized that by simultaneously activating p110 α with p110 γ we would attenuate the maladaptive cardiomyopathy effects associated with the gamma-subtype. Here, we observed the differential expression of p110 α and p110 γ in each of the treatment groups (Figure 4). Specifically, in the Iso-treated group, only p110 γ

was significantly increased, while IGF-1 treatment alone significantly increased p110 α expression with no increase in p110 γ . As we predicted, in the Iso+IGF-1 group both p110 α and p110 γ were concurrently increased (Figure 4A & 4B). Activation of Akt-dependent signaling downstream of p110 α is known to play a critical role in mediating the adaptive hypertrophic response. Here we observe no change in total Akt expression (Figure 4C) but a significant increase in activated (phosphorylated) Akt in the Iso+IGF-1 and IGF-1 groups (Figure 4D). These data indicate that despite chronic sympathetic drive, IGF-1 may preferentially activate cardioprotective and physiological hypertrophic signaling cascades.

IGF-1 treatment promotes BH4 production

Inducible nitric oxide synthase (iNOS) and its uncoupling from NO production to reactive oxygen species (ROS) is associated with the development of HF (Haywood et al., 1996, Silberman et al., 2010, Ziolo et al., 2004, Ziolo et al., 2008). As expected we observed a >3-fold increase in iNOS expression hearts treated only with Iso. However, we found that iNOS expression was increased ~4-fold in rats treated with Iso+IGF-1 (Figure 5A). This was unexpected given the maintained cardiac performance of these rats. The uncoupling of NOS from NO production to ROS production is in part mediated by the loss of the NOS co-factor, tetrahydrobiopterin (BH4) (Silberman et al., 2010, Xia et al., 1998). This uncoupling is expected to promote an oxidized environment and lead to myocardial damage. Using HPLC, we observe a significant decrease in BH4 and total biopterin levels in Iso-treated hearts. This loss of BH4 and biopterin was completely reversed by IGF-1 treatment (Figures 5B and 5D). Since GTP cyclohydrolase 1 (GTPCH1) is the rate-limiting enzyme in the *de novo* synthesis of BH4, we measured myocardial GTPCH1 expression by western blot. Figure 5C shows that GTPCH1 expression was significantly increased in hearts treated with Iso, likely a compensatory mechanism for decreased BH4. Interestingly, GTPCH1 expression in Iso +IGF-1 hearts was no different than control. This is the first report to indicate that IGF-1-dependent signaling regulates BH4 production in the heart.

Discussion

This study provides new *in vivo* functional data and a potential molecular mechanism regarding the therapeutic impact of IGF-1 on the development of cardiac dysfunction in the setting of chronic β -adrenergic stimulation. Over the time course of this study, rats treated with Iso alone developed cardiac hypertrophy with diastolic and systolic dysfunction as measured by PV loops, echocardiography, and ECG. A simple concurrent treatment with daily SQ IGF-1 in rats chronically perfused with Iso resulted in significant improvements of the measured diastolic and systolic parameters. The most unexpected finding of this study was the ability of IGF-1 treatment to maintain adrenergic responsiveness of the heart despite the presence of chronic adrenergic stimulation. Decades of research has repeatedly demonstrated that chronic catecholamine stimulation leads to a desensitization of the adrenergic system in the heart, and this desensitization is a common feature of human heart failure (Rockman et al., 2002).

The preservation of adrenergic responsiveness and increases in cardiac performance are found in athletic hypertrophy, and recent data has indicated that exercise promotes

maintained adrenergic response in multiple preclinical models of heart failure (Leosco et al., 2008, MacDonnell et al., 2005, Serra et al., 2008, Roof et al., 2013). Relevant to this study, new data has emerged indicating that BH4 and GTPCH1, in part, regulate adrenergic sensitivity (Adlam et al., 2012). BH4 is an essential cofactor of nitric oxide synthases. As BH4 levels decrease, NOS becomes uncoupled from NO production and favors the production of ROS, such as superoxide (Alkaitis and Crabtree, 2012). This increase in cellular ROS leads to cell damage and promotes the oxidation of many key proteins involved in excitation-contraction coupling in the heart, supplying an arrhythmogenic substrate (Ziolo and Houser, 2014, Roof et al., 2015). While ROS production per se was not measured here, maintained levels of BH4 would be expected to recouple NOS to NO production and are indicated to be cardioprotective (Szelenyi et al., 2015).

Uncoupling of NOS has been linked with the development of diastolic dysfunction (Silberman et al., 2010). For these reasons, targeting BH4 production has recently emerged as a potential therapeutic target for patients with heart failure with preserved ejection fraction (or diastolic failure) (Carnicer et al., 2012, Moens and Kass, 2007, Jeong et al., 2013). Here, we show that BH4 levels are normalized along with diastolic function by treatment with IGF-1 (Figure 5). While IGF-1 has been linked with BH4 production in other tissue types (Elzaouk et al., 2003, Tanaka et al., 2002), this is the first report indicating IGF-1 regulates BH4 production in the heart. We speculate that the IGF-1-dependent effects on BH4 levels in the heart are, in part, mediating the effects we observe on the development of diastolic dysfunction. It is an intriguing possibility that the beneficial effects of exercise therapy for patients with heart failure may, in part, be mediated by increased IGF-1 production leading to IGF-1-dependent changes in BH4 and NOS-dependent signaling.

Approximately 50% of patients hospitalized for HF are diagnosed with diastolic failure. Furthermore, in patients hospitalized for systolic failure the presence and severity of diastolic dysfunction is predictive of all-cause mortality (Halley et al., 2011). There are no therapies indicated for diastolic failure. Our new data indicate that IGF-1 is exerting pleiotropic effects on the heart and cardiovascular system, and these effects are modulating not only diastolic function but systolic function as well. We find the diminished systolic function resulting from Iso treatment was significantly improved by treatment with IGF-1. In particular, CO, dp/dt_{max} , and PRSW were all significantly enhanced (Figure 3).

The underlying signaling mechanisms mediating adaptive and maladaptive hypertrophy are complex. While the current body of data points to p110 α and p110 γ mediating discrete hypertrophic responses, our understanding of these mechanisms is incomplete. It is generally believed that the activation of p110 α is linked with the development of athletic hypertrophy, while p110 γ mediates maladaptive remodeling. Yet, the current study and other published data indicate that these two signaling pathways likely affect one another through some level of crosstalk (McMullen et al., 2007, McMullen et al., 2003). In a series of elegant studies, McMullen et al. demonstrated that activation of p110 α is required for exercise-induced, adaptive hypertrophy (McMullen et al., 2003). Further work by this group demonstrated in a mouse model with increased p110 α activity, the progression of pathological p110 γ -linked hypertrophy induced by G protein-coupled receptor stimulation and dilated cardiomyopathy was slowed or completely arrested (McMullen et al., 2007). However, it is important to note

that chronic activation of either IGF-1- or adrenergic-dependent signaling alone leads to maladaptive hypertrophy (i.e. acromegaly or HF, respectively). This implies that while the exclusive activation of either p110 α or p110 γ alone is detrimental, the two may synergize to result in adaptive remodeling, as our data suggest. Here we show that the expected functional outcome resulting from chronic adrenergic stimulation has been altered, blunted, or is altogether different with concurrent IGF-1 treatment. Indeed, we observe the differential activation of hypertrophic signaling cascades (Figure 4). We find that in rats treated with Iso, p110 γ signaling is increased and leads to maladaptive hypertrophy. However, in rats treated with Iso+IGF-1 both p110 γ and p110 α signaling are increased. Activated Akt (a cardioprotective signal) is also found in rats treated with IGF-1. This data suggests that treatment with IGF-1 is able to alter the underlying signaling cascades associated with adrenergic stimulation. Further work is needed to determine the precise molecular players mediating the differential hypertrophic outcomes.

The pleiotropic effects of IGF-1 extend to the circulatory system as well. As expected, pulse wave velocity and MAP were increased by Iso treatment, indicative of decreased vascular compliance. However, the simultaneous treatment with Iso+IGF-1 normalized both these parameters (Fig.1). IGF-1 increases the release of nitric oxide from endothelial cells and lowers BP (Tsukahara et al., 1994). This IGF-1-dependent effect would normalize the increased afterload resulting from chronic circulating catecholamines and relieve the hemodynamic stress placed upon the heart. This in part explains the differences in LV chamber size and ejection fraction between rats treated with Iso and those treated with Iso +IGF-1 (Table 1). However, it is important to note that these changes in BP are coupled to IGF-1-dependent effects on myocardial performance. Directly assessing BP-dependent from BP-independent effects in our experimental approach is difficult. High circulating catecholamines are known to act directly on the myocardium and produce fibrosis (Bos et al., 2005). We observed a significant attenuation of fibrosis with IGF-1 treatment (Figure 1), suggesting a direct of IGF-1 on the myocardium. Furthermore, functional parameters such as PRSW, ESPVR, EDPVR, and the E/A ratio are all independent of preload and afterload (Burkhoff et al., 2005). Therefore, the PV loop data indicate that IGF-1 is having direct effects on myocardial performance (Figures 2 and 3).

Hypertrophic remodeling was observed and expected in both the Iso and Iso+IGF-1 treated rats. However, the hypertrophic fates of these two groups are divergent. Importantly, the dilatation of the ventricle observed in the Iso treated rats was not present in those treated with Iso+IGF-1, and ejection fraction was completely preserved. Furthermore, adrenergic responsiveness was preserved in the Iso+IGF-1 group. Taken together, these data suggest that while still hypertrophic the phenotype resulting from the simultaneous application of Iso +IGF-1 shifts the structural remodeling and hemodynamics more towards that observed in physiological hypertrophy (Figure 6).

We speculate that in the setting of pre-existing HF where circulating catecholamines are increased, activation of p110 α would promote a more physiological cardiac remodeling. Indeed, this shift in hypertrophic remodeling is observed in the clinic as HF patients undergo strict exercise therapy. Using IGF-1 as a surrogate for exercise in an immobile patient population may exert similar effects. This intervention would not require sophisticated

therapeutic approaches (such as gene therapy, or the application of drugs designed to target p110 α/γ). IGF-1 is already approved for clinical use in children with growth hormone deficiency. Thereby, the transition to use in a HF population would be eased. This repurposing of IGF-1 makes it attractive as the development of novel effective therapies against HF, like the angiotensin receptor-neprilysin inhibitor, LCZ696, remains slow (McMurray et al., 2014).

While a handful of small clinical trials investigating IGF-1 as a therapy for various forms of HF have been conducted, all have been underpowered and had major variations in IGF-1 dosage. These technical problems within these studies make a consensus difficult to reach. However, meta-analysis of all available clinical trials indicates that IGF-1 treatment significantly improves cardiac function in patients with HF (Le Corvoisier et al., 2007). Notably, no trials have systematically examined the impact of IGF-1 on diastolic failure (Perkel et al., 2012). Our data adds to this developing story by providing new insights into the effect of IGF-1 on diastolic dysfunction and how it may preserve β -AR responsiveness under the condition of chronic sympathetic tone.

Limitations

Our results indicate that applying IGF-1 significantly attenuates the development of diastolic and systolic dysfunction when initiated concurrently with Iso treatment. The ability to rescue cardiac performance after dysfunction has already developed was not addressed by this study. This was an original aim of this study. However, in the summer of 2013 a global shortage of IGF-1 began due to production difficulties encountered by the only manufacturer of this drug. Currently, the availability and cost of Increlex to researchers severely limits further studies at this time. Acute cellular studies and, especially, those requiring long term IGF-1 therapy—such as survival studies—are both infeasible until this shortage is alleviated. However, the data presented here strongly suggest the positive impact that IGF-1 therapy may be expected to have on long term survival in this model of cardiomyopathy. Importantly, two previous studies have reported that IGF-1 treatment applied after myocardial infarct positively affected cardiac performance and survival, indicating that IGF-1 treatment may rescue cardiac performance after the onset of dysfunction (Duerr et al., 1996, Cittadini et al., 1997).

Conclusion

We find that in the setting of cardiomyopathy induced by chronic adrenergic tone in rats, treatment with daily SQ injection of IGF-1 prevented the development of diastolic dysfunction, maintained adrenergic responsiveness, partially preserved systolic function, and completely attenuated the observed increase in MAP. We demonstrate that p110 α and p110 γ are simultaneously increased along with activated Akt by treatment with Iso+IGF1. Furthermore, we show that that IGF-1-dependent signaling promotes BH4 production in the heart. This new data and the growing body of evidence indicate that further clinical investigations into IGF-1 as a potential therapy for HF are warranted.

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Figure 1. Histopathology and hemodynamics

Masson's trichrome stained tissue sections from after 2 weeks (**A**) and 4 weeks (**B**) of treatment. (**C**) Fibrosis at 4 weeks was significantly increased in the Iso group compared to all others. (**D**) No differences in apoptosis, as measured by cells positively stained for cleaved caspase-3, were observed amongst the treatments. (**E**) Iso treatment significantly increases blood pressure, and this effect is normalized by IGF-1 treatment. (**F**) Pulse wave velocity is significantly increased by Iso treatment. (* $p < 0.05$ vs control, + $p < 0.05$ vs. Iso + IGF-1, $n = 5-7$)

Figure 2. Diastolic dysfunction is attenuated by IGF-1 treatment

(A) Representative families of curves demonstrating the EDPVR in each treatment. (B) Summary statistics of the slope of EDPVR demonstrating that Iso+IGF-1 normalized this relationship. (C) The rate corrected QT interval is prolonged by Iso treatment but normal in the Iso+IGF-1 group. (D) The E/A ratio is shifted in favor of atrial filling in Iso-treated rats. (E) End diastolic pressure is significantly increased by Iso treatment. This Iso-dependent effect was reversed in the Iso+IGF-1 group. (F) The significant increase in end diastolic pressure in the Iso group is maintained in response to dobutamine. The response of the Iso +IGF-1 group was normal. (G) The tau or relaxation is significantly longer at baseline and after stimulation with dobutamine in Iso-treated hearts but is attenuated in the Iso+IGF-1 group, likely contributing to decreased diastolic filling. (H) Minimum rate of pressure change is significantly increased by treatment with Iso but attenuated by treatment with IGF-1. (* $p < 0.05$ vs control, + $p < 0.05$ vs. Iso+IGF-1, $n = 6$, Two way ANOVA panels F-H)

Figure 3. Systolic dysfunction is attenuated by IGF-1 treatment

(A) Representative PV loops from each treatment demonstrating the decreased ESPVR in the Iso-treated rats and its attenuation by IGF-1 treatment. For clarity, the family of loops generated to establish the ESPVR are not shown. (B) Summary statistics of the slope of ESPVR indicate impair contractility in Iso-treated hearts with attenuation of this effect by Iso+IGF-1 treatment. (C) Preload recruitable stroke work is decreased by Iso treatment, but partially reversed in hearts treated with Iso+IGF-1. (D) Iso-treated hearts are unable to significantly increase cardiac output in response to β -AR stimulation unlike those treated with Iso+IGF-1. (E) The increased heart rate after β -AR stimulation is significantly blunted in the Iso-treated group but is preserved in the Iso+IGF-1 group. (F) The maximum rate of pressure change in response to β -AR stimulation is blunted by Iso treatment and partially attenuated in heart treated with Iso+IGF-1. (G) Arterial elastance is significantly increased at baseline and during dobutamine stimulation in the Iso treated group. No differences were observed in the Iso+IGF-1 group (* $p < 0.05$ vs. control, + $p < 0.05$ vs. Iso+IGF-1, $n = 6$)

Figure 4. IGF-1 treatment promotes cardioprotective signaling

(A) In hearts treated with Iso, p110 γ is significantly increased. (B) Treatment with IGF-1 induces a significant increase in p110 α expression. Both isoforms are increased in the Iso + IGF-1 group. (C) Expression level of Akt is unchanged in all groups compared to control. (D) An increased ratio phosphorylated-Akt to total Akt was observed in the IGF-1 and the Iso + IGF-1 group. (* $p < 0.05$ vs. control, $n = 6$)

Figure 5. Myocardial BH4 content is rescued by IGF-1 treatment in ISO-treated hearts
(A) Increased iNOS expression is found in both ISO and ISO + IGF-1 treatment groups compared to control and IGF-1 treatment groups. **(B)** Myocardial levels of BH4 are significantly lower in the ISO-treated group, but are similar to Control in the ISO + IGF-1-treated group. **(C)** GTPCH1 expression is increased in hearts treated with ISO. **(D)** Overlay of representative chromatograms showing elution of BH4 peak in all groups. The BH4 standard chromatogram is inset. (* $p < 0.05$ vs. control, $n = 6$)

Figure 6. Central illustration of IGF-1 treatment in shifting pathological hypertrophy towards athletic hypertrophy

Table 1**Structural Parameters**

Echo Parameter	Control	IGF1 alone	Iso alone	Iso+IGF1
IVS_d , baseline (mm)	1.60±0.08	1.60±0.04	1.40±0.05	1.50±0.06
2 weeks	1.64±0.07	1.78±0.10	1.83±0.19	1.93±0.10 ^{*†}
4 weeks	1.76±0.14	1.84±0.09	2.07±0.13 ^{*†}	2.13±0.20 ^{*†}
IVS_s , baseline (mm)	2.28±0.09	2.27±0.05	2.14±0.04	2.21±0.08
2 weeks	2.35±0.14	2.60±0.13	2.36±0.26	2.81±0.30
4 weeks	2.58±0.18	2.63±0.16	2.68±0.17 ^{*†}	3.01±0.31 ^{*†}
LVID_d , baseline (mm)	7.60±0.14	7.40±0.23	7.50±0.02	7.80±0.13
2 weeks	7.14±0.57	7.43±0.83	7.78±0.52	7.97±0.44
4 weeks	7.35±0.56	7.39±0.11	8.44±0.34 ^{*†}	7.76±0.50
LVID_s , baseline (mm)	4.93±0.18	4.95±0.24	5.10±0.14	5.36±0.15
2 weeks	4.46±0.44	4.54±0.60	5.44±0.41	4.70±0.38
4 weeks	4.53±0.40	4.46±0.20	5.81±0.28 [*]	4.91±0.39
LVPW_d , baseline (mm)	1.68±0.09	1.69±0.6	1.65±0.03	1.76±0.07
2 weeks	1.72±0.17	1.78±0.16	1.76±0.16	1.85±0.31
4 weeks	1.75±0.24	1.83±0.09	1.75±0.25	1.90±0.18
LVPW_s , baseline (mm)	2.44±0.11	2.41±0.07	2.25±0.05	2.37±0.10
2 weeks	2.49±0.18	2.59±0.18	2.40±0.17	3.15±0.35
4 weeks	2.61±0.38	2.77±0.26	2.70±0.34	2.79±0.23
EF , baseline (%)	71.8±3.8	70.0±2.7	68.6±3.2	68.3±4.8
2 weeks	75.2±3.0	76.8±2.9	65.6±2.0 ^{*‡}	77.8±1.7
4 weeks	76.6±1.4	77.9±3.7	64.5±3.4 ^{*‡}	75.1±1.4
HW/BW (mg/g)	n/a	n/a	n/a	n/a
2 weeks	3.01±0.065	3.31±0.072 [*]	3.49±0.080 ^{*‡}	3.85±0.053 [*]
4 weeks	3.45±0.083	3.62±0.10	3.88±0.105 [*]	3.77±0.093 [*]
n baseline	7	5	5	5
2 weeks	7	7	6	6
4 weeks	6	6	6	6

IVS_d, diastolic interventricular septum thickness; IVS_s, Systolic interventricular septum thickness; LVID_d, diastolic left ventricular inner diameter; LVID_s, systolic left ventricular inner diameter; LVPW_d, diastolic left ventricular poster wall thickness; LVPW_s, systolic left ventricular poster wall thickness; EF, ejection fraction

* p<0.05 vs. control baseline,

† p<0.05 vs. baseline,

‡ p<0.05 vs. Iso+IGF1