Title: Age exacerbates chronic catecholamine-induced impairments in contractile reserve in the rat.

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Abstract

Contractile reserve decreases with advancing age and chronic isoproterenol (ISO) administration is a well-characterized model of cardiac hypertrophy known to impair cardiovascular function. This study evaluated whether non-senescent, mature adult rats are more susceptible to detrimental effects of chronic ISO administration than younger adult rats. Rats received daily injections of ISO (0.1 mg/kg, s.c.) or vehicle (V) for three weeks. ISO induced a greater impairment in contractile reserve ($\Delta +dP/dt_{max}$) in mature adult ISO-treated (MA-ISO) than in young adult ISO-treated rats (YA-ISO) in response to infusions of mechanistically distinct inotropes (digoxin, milrinone; 20 – 200 $\mu$l/kg/min), while basal and agonist-induced changes in heart rate and SAP were not different across groups. ISO decreased expression of the calcium handling protein, SERCA2a, in MA-ISO compared to YA, YA-ISO and MA rats. Chronic ISO also induced greater increases in cardiac hypertrophy (LV index: 33±3 % vs. 22±5 %) and caspase-3 activity (34 % vs. 5%) in MA-ISO relative to YA-ISO rats. Moreover, $\beta$-MHC and ANF mRNA expression was significantly elevated in MA-ISO. These results demonstrate that adult rats develop greater impairments in systolic performance than younger rats when exposed to chronic catecholamine excess. Reduced contractile reserve may result from calcium dysregulation, increased caspase-3 activity, or increased $\beta$-MHC and ANF expression. Although several studies report age-related declines in systolic performance in older and senescent animals, the current study demonstrates that catecholamine excess induces reductions in systolic performance significantly earlier in life.

Key Words: cardiac hypertrophy, contractile reserve, age, isoproterenol
**Introduction**

It is well established that older patients, when subjected to increases in sympathetic drive and/or afterload, develop heart failure more frequently compared to younger populations (21, 23, 33, 36). Additionally, incidences of cardiovascular disease, cardiac hypertrophy, and heart failure (HF) are more prevalent with advancing age (7, 8). The aging heart, in both humans and rats, becomes functionally impaired at the level of the organ and the cardiomyocyte (7, 21, 33). Age-related decreases in the contractile reserve of the heart are associated with left ventricular (LV) hypertrophy, activation of pro-apoptotic enzymes, and calcium cycling defects within the myocyte (2, 9, 19, 22). The underlying cause of age-related declines in systolic performance are not completely understood but may include the following: a decrease in crucial Ca$^{2+}$ handling proteins such as SERCA2a, a shift in myosin gene expression that results in higher levels of low-velocity β-Myosin heavy chain (β-MHC), or an increase in caspase activity. (1, 7-10, 21, 22, 33).

Chronic administration of the non-selective β-receptor agonist isoproterenol (ISO) is an experimental model of cardiac hypertrophy that is characterized by decreases in cardiac contractility, disruptions in intracellular Ca$^{2+}$ handling, and increases in cardiomyocyte apoptosis and necrosis (6, 32, 51). Chronic increases sympathetic activity and the sustained elevation of circulating catecholamines are known to worsen systolic function and to decrease contractile reserve, whereas β-receptor antagonism mitigates the progression of clinical heart failure (31, 36).

A blunted contractile reserve is indicative of decreased myocardial viability and characteristic of human HF (5, 44). Inotrope-induced increases in +dP/dt$_{Max}$ reflects
contractile reserve, and this index is measured to clinically assess a patient’s cardiac performance (26, 44). Cardiac function can often appear normal until subjected to stresses, such as excessive adrenergic stimulation, which reveal underlying impairments (23, 36). In fact, an ample contractile reserve is predictive of a better prognosis in patients with LV dysfunction at rest (18).

Because mature adults may have a diminished capacity to respond to cardiac stress compared to those in the growing phase, and because aging and catecholamine excess negatively impact cardiovascular function, the present study was undertaken to determine whether mature adult rats are more susceptible to ISO-induced reductions in contractile reserve than younger adult rats. Previous reports on the effect of aging on cardiovascular function in rats have focused primarily on comparisons between senescent or old (> 20 mos) and mature adult (2-4 mos) animals (1). These studies have revealed that older rodent hearts have depressed contractility as measured by changes in +dP/dt_{Max}, and have reduced levels of SERCA2a (1, 13). While the senescent, aging heart is more susceptible to cardiac insult, little is known concerning the difference in cardiac functional responses between mature adult and young adult rats following chronic catecholamine excess (10, 19). The present study was conducted in groups of animals not yet expected to demonstrate age-related detriments in myocardial structure or resting function (7, 8). The animals selected for the current experiments represent precise and distinct stages of development according to the lifespan and growth of this particular species and strain (12). Animals identified as Young Adults (YA; aged 12 weeks at endpoint) were still in their rapidly growing phase while those identified as Mature Adults (MA; aged 18 weeks at endpoint) had reached expected maximal weight when functional measurements were
recorded (12). This assertion is supported by the fact that YA animals in the present study experienced a 43% increase in weight while MA increased in weight by only 15%. Over the course of the 3 week study, YA grew from 240 ± 4g (9 wks old) to 344 ± 7g (12 wks old) while MA grew from 399 ± 3g (15 wks old) to 460 ± 5g (18 wks old).

**Materials and Methods**

*Animals.*

All experimental procedures were carried out in accordance with protocols approved by the Gilead Colorado Institutional Animal Care and Use Committee. Male Sprague Dawley rats (Crl:SD; Charles River Laboratories International, Inc.) were maintained on a standard laboratory diet (Teklad 22-5, 8640) with water *ad libitum*. Experiments were performed on young adult (YA; aged 9 weeks at study inception and aged 12 weeks at endpoint) and mature adult (MA; aged 15 weeks at study inception and aged 18 weeks at endpoint). Initial pilot studies established that increases in HR following ISO (0.1 mg/kg, s.c.) were similar in MA and YA (140 ± 15 vs. 150 ± 25 bpm). Following a one week acclimation period, animals were separated into four weight and age-matched groups: young adults that received daily injections of the vehicle (1% ascorbic acid solution in DiH₂O) for 3 weeks (YA); young adults that received daily injections of ISO (0.1 mg/kg/day, s.c.) for 3 weeks (YA-ISO); mature adult animals that received daily injections of the vehicle for 3 weeks (MA); and mature adult animals that received daily injections of ISO (0.1 mg/kg/day, s.c.) for 3 weeks (MA-ISO). All animals were weighed daily between 08:00 and 09:30, and dosed immediately thereafter to ensure consistent, weight-dependent administration of drugs and vehicle throughout the study. Animals did
not receive injections on the day hemodynamics and cardiac performances were evaluated.

In Vivo Ventricular and Systemic Hemodynamics.

Anesthesia was induced by inhalation of isofluorane in a closed chamber. Animals were then intubated and the appropriate plane of anesthesia was maintained during the entire course of the experiment with 2% isofluorane delivered through a vaporizer (VIP 3000; Matrx, Orchard Park, NY) and carried by 100% oxygen (0.8 l/min). Peak inspiratory pressure was maintained between 12-15 cmH₂O and the ventilation rate was kept at 50bpm (Anesthesia Workstation; Halloewell EMC, Pittsfield, MA). A Millar catheter (SPR-869, Millar Instruments, Houston, TX) was inserted into the right carotid artery and advanced into the left ventricle through the aortic valve. A separate Millar catheter (SPR-838) was inserted into the right femoral artery and advanced into the abdominal aorta for measurement of systemic hemodynamics. A segment of PE-50 was inserted into the right femoral vein for the infusion of drugs. A rectal thermometer attached to a servo-controlled heating pad (TC-1000; CWE Inc., Ardmore, PA) was used to maintain body temperature at 37 ± 0.1°C. After allowing for stabilization from surgical preparation, steady-state left ventricular and systemic hemodynamics were recorded on a PC using a MPVS-300 pressure-volume unit (ADInstruments Inc., Colorado Springs, CO). All hemodynamic data were acquired with the ventilator briefly turned off (3-5 sec). Tau (Weiss), a time constant of LV pressure decay, was used to assess LV diastolic function. Increases in Tau (Weiss) occur with prolonged relaxation times and are attributed to a reduction in the rate of diastolic removal of cytoplasmic Ca²⁺ via
SERCA2a (22). PVAN data analysis software (v3.5; Millar Instruments, Houston, TX) was used to analyze all ventricular and systemic hemodynamic data.

Assessment of Contractile Reserve

The maximum rate of left ventricular pressure development (+dP/dt\textsubscript{Max}) following inotrope and/or vehicle infusions was compared to steady-state baseline values for each animal to determine Delta +dP/dt\textsubscript{Max}. The Delta +dP/dt\textsubscript{Max} accurately reflects systolic performance and was used as an index of contractile reserve (26). In fact, estimates of +dP/dt\textsubscript{max} can reveal functional impairments in asymptomatic and mildly symptomatic HF patients who have normal LV performance at rest (16, 28). Two distinct pharmacological agents were used to assess contractile reserve, and animals were randomly assigned to a given agent prior to study inception. Increasing doses (20, 60, then 200 μg/kg/min; μkm) of milrinone or digoxin were serially infused (i.v.) based on dose-response curves previously generated in naïve young and adult animals (data not shown). Due to solubility limitations, lower doses (20 and 60 μkm) were infused at a rate of 10 μl/min per 100g body weight (e.g. 300g rat received 30 μl/min) while the highest dose of agonist (200 μkm) was infused at 20 μl/min per 100g body weight. Pilot studies demonstrated that this increment in infusion rate does not elicit effects on ventricular or systemic hemodynamics over the course of a typical experiment. Steady-state ventricular and systemic parameters were recorded prior to infusion of drug. Infusions continued for 10-15 minutes and data was recorded after hemodynamics and +dP/dt\textsubscript{Max} were stable.

General Experimental Protocol
After three weeks of daily ISO or vehicle administration, a subset of each treatment group was subjected to contractile reserve assessment with either digoxin or milrinone. Cardiac hypertrophy was assessed by calculating the left ventricular index (LVI) for each animal according to the following formula: \[\text{weight of LV free wall with septum (mg) / tibia length (mm)}\]. To control for the potential effects of inotropic agents on biochemical endpoints, separate groups of rats received either vehicle or ISO for three weeks prior to being subjected to endpoint biochemical analysis. To ensure similar disease phenotype, baseline ventricular and hemodynamic parameters, as well as morphologic data (LVI, body weight) were recorded in every animal.

Quantitative Real Time PCR

Total RNA was isolated from tissue by using a Ribopure Kit (Ambion, Austin, TX) and 1 μg was reverse transcribed using the Superscript III cDNA Synthesis kit (Invitrogen Corp. Carlsbad CA) according to manufacturer’s protocol. Quantitative RT-PCR was done on an ABI 7300 Real Time PCR instrument (Applied Biosystems, Foster City, CA) using Taqman Universal PCR Master Mix (Applied Biosystems) following the manufacturer’s instructions. Probes and primers were from TaqMan Gene Expression Assay reagents (SERCA2A-Rn00568762_m1; Applied Biosystems). Standard curves were generated by serial dilution of cDNA, and relative quantification of target and housekeeping gene levels were done by ABI 7300 instrument algorithm software. Relative levels of target RNA between samples was calculated after normalization with 18S ribosomal RNA (rRNA) gene (cat# 4308329).
QuantiGene Multiplex Assays for mRNA Expression

A 10-30 mg sample of apical LV free wall was homogenized in Tissue & Cell Lysis Buffer (Epicentre Technologies, Madison, WI) supplemented with 100 μg/ml Proteinase K (Panomics, Fremont, CA) for 2 hours at 60°C with occasional shaking. Homogenates were then used in the QuantiGene Multiplex Assay (Panomics) by hybridizing specified probe sets according to manufacturer’s instructions. Transcript expression was subsequently measured in a Luminex® 200™ (Luminex Corp., Austin TX) and relative quantitation of mRNA levels was calculated after normalization against the house keeping gene, HPRT (hypoxanthine phosphoribosyltransferase).

Caspase Activity

A caspase-3 fluorometric assay kit (BF1100; R&D Systems Inc, Minneapolis, MN) and a BCA Protein Assay Kit (23235; Thermo Fisher, Rockford, IL) were used in the current study. A tissue lysis buffer containing 1X PBS, 1mM EDTA, 0.5% Triton x100, 1x phosphatase inhibitor (Thermo Fisher 78428), and 1x protease inhibitor (Thermo Fisher 1860932) was prepared. Uniform samples of apical LV free wall were lysed in 500 μl of tissue lysis buffer and BCA assay was performed to determine the total protein concentration. Caspase-3 fluorometric assays were performed in 96-well flat bottom microplates. The reaction mixture contained 50 μl of tissue lysate (200 μg total protein), 50 μl of assay buffer 2X, and 50mM of fluorescent substrate. Plates were incubated at 37°C for 1 hour and read on a SpectroMax M5e (light excitation at 400nm with emitted light at collected 505nm). The results are expressed as fold increase in caspase activity normalized by the control group.
Drugs

(−)-Isoproterenol hydrochloride, milrinone, digoxin, and (+)-Sodium L-ascorbate were obtained from Sigma-Aldrich (St. Louis, MO). Isoproterenol, milrinone, and ascorbic acid were dissolved in diH₂O, whereas digoxin was dissolved in a solution of 33% methanol and diH₂O, which was then diluted to achieve lower doses.

Data analysis and statistics.

A One-way ANOVA followed by Bonferroni multiple comparisons test was performed on baseline and endpoint data to determine the effect of ISO treatment. These results were analyzed on a PC (GraphPad Prism version 5.0; GraphPad Software, Inc) and presented as means ± SE. A value of $P \leq 0.05$ was considered statistically significant. Data generated during serial infusions of an inotrope were analyzed using two-way repeated measures ANOVA with a Holm-Sidak post-test on a PC (SigmaPlot version 11.0; Systat Software, Inc) and presented as means ± SE. A value of $P \leq 0.05$ was considered statistically significant.
Results

Effect of chronic isoproterenol administration on baseline cardiac performance and hemodynamics

The effect of chronic ISO administration (0.1 mg/kg, s.c., for 21 days) on baseline ±dP/dt (mmHg*sec⁻¹), Tau (Weiss; mSec), heart rate (bpm), and mean arterial pressure (MAP; mmHg) was investigated in YA and MA rats, and these data are summarized in Figures 1 and 2. There was no difference in baseline contractility between YA-ISO, MA, and MA-ISO rats. However, YA rats had significantly greater positive and negative basal dP/dtMax (6812 ± 264 and -8091 ± 355 mmHG x sec⁻¹) as compared to YA-ISO (5089 ± 223 and -5583 ± 200 mmHG x sec⁻¹), MA (5841 ± 239 and -6249 ± 350 mmHG x sec⁻¹), and MA-ISO (5356 ± 197 and -5960 ± 369 mmHG x sec⁻¹), (Fig 1). Basal ventricular relaxation as indexed by Tau (Weiss, mSec) was similar in YA and MA controls (10.36 ± 0.36 vs 10.18 ± 0.34) (Fig 2A). Chronic ISO significantly increased LV relaxation time in both YA-ISO and MA-ISO compared to their age-matched vehicle controls (12.89 ± 0.51; 12.07 ± 0.32, respectively) (Fig. 2A). There were no differences in basal heart rate or MAP between treatment groups (Figs. 2B-C).

Effect of chronic isoproterenol administration on responses to mechanistically distinct inotropic agents

Responses to infusions of milrinone were investigated in young and mature adult animals following chronic ISO administration (Figure 3A-B). ISO treatment did not significantly influence the effect of serial milrinone infusions (20 – 200 μg/kg/min) on increases in +dP/dtMax in YA-ISO compared to YA controls (P = 0.052). However, the response to the
middle dose of milrinone (60 μg/kg/min) was statistically depressed in YA-ISO compared to YA controls (P = 0.007) whereas responses following low (20 μg/kg/min) and high (200 μg/kg/min) doses of milrinone were not different (P = 0.068 and P = 0.658, respectively) compared to YA (Fig 3A). In contrast, the effect of serial milrinone infusions (20-200 μg/kg/min) on changes in +dP/dt\text{Max} was attenuated in MA-ISO compared to vehicle treated, age-matched controls (P = 0.001)(Fig 3B). Moreover, increases in +dP/dt\text{Max} were decreased at every dose of milrinone (20, 60, 200 μg/kg/min) (P = 0.039, P = 0.001, and P < 0.001, respectively.) in MA-ISO compared to MA controls (Fig 3B). Milrinone (20-200 μg/kg/min) increased +dP/dt\text{Max} similarly in YA and MA controls (P > 0.5) (Fig 3A-B). HR, SAP, and MAP were similar across groups following the infusion of milrinone (Table 1).

Responses to infusions of increasing doses of digoxin (20 – 200 μg/kg/min) were investigated in young adult and mature adult animals following chronic ISO administration (Fig 4A-B). ISO treatment did not significantly influence the effect of serial digoxin infusions (20 – 200 μg/kg/min) on increases in +dP/dt\text{Max} in YA-ISO compared to YA controls (P = 0.194) (Fig 4A). Responses in YA-ISO were decreased following the 200 μg/kg/min infusion compared to YA (P = 0.008), whereas responses at low (20 μg/kg/min) and middle (60 μg/kg/min) doses of milrinone were not different (P = 0.926 and P = 0.154, respectively) (Fig 4B). In contrast to what was observed in young adults, ISO treatment significantly attenuated the effect of digoxin (20 – 200 μg/kg/min) on increases in +dP/dt\text{Max} in MA-ISO compared to MA controls (P = 0.01) (Fig 4B). Moreover, increases in +dP/dt\text{Max} were diminished at every dose of digoxin (20, 60, 100 μg/kg/min) (P < 0.001, P = 0.042, and P < 0.036, respectively.) in MA-ISO compared to
MA (Fig. 4B). Digoxin infusions (20 – 200 μg/kg/min i.v.) induced similar increases in 
$+dP/dt_{\text{Max}}$ in YA and MA ($p > 0.5$) (Fig 4A-B). HR, SAP, and MAP were similar across 
groups following the infusion of digoxin (Table 1).

**Effect of chronic isoproterenol administration on left ventricular index (LVI)**

The effects of chronic ISO administration on cardiac hypertrophy are shown in figure 5. Chronic ISO administration induced significant increases in LV hypertrophy (LVI) in both YA-ISO and MA-ISO rats compared to their age-matched vehicle controls (YA: $24.6 \pm 1.0$ vs. $20.2 \pm 0.8$; MA: $28.0 \pm 0.6$ vs. $21.2 \pm 0.5$) ($P < 0.05$) (Fig 5). However, ISO induced a greater increase in LVI in MA-ISO compared to YA-ISO rats ($33 \pm 3 \%$ vs. $22 \pm 5 \%$ increase) (Fig. 5).

**Effects of chronic isoproterenol and age on biochemical indices of LV contractility and pathological remodeling**

Quantitative PCR was used to investigate changes in LV expression of SERCA2a (Fig. 6). MA had reduced SERCA2a mRNA expression compared to YA rats ($91 \pm 18$ vs. $161 \pm 11$ arbitrary units, 18S corrected) ($P < 0.05$). ISO treatment diminished SERCA2a mRNA expression in both YA-ISO and MA-ISO animals compared to their respective age-matched vehicle controls (YA: $82 \pm 15$ vs. $161 \pm 11$; MA: $36 \pm 4$ vs. $91 \pm 18$) ($P < 0.05$). Furthermore, MA-ISO rats had reduced SERCA2a mRNA expression compared to YA-ISO rats ($36 \pm 4$ vs. $82 \pm 15$) ($P < 0.05$) (Fig. 6). The effect of ISO on caspase-3 activity in LV tissue was also investigated. There were no differences in caspase activity detected amongst YA, YA-ISO and MA rats. In contrast, chronic ISO administration
increased caspase activity 1.34 fold in MA-ISO compared to MA controls ($P < 0.05$) (Fig 7).

The effect of chronic ISO administration on LV $\beta$-MHC, ANF, and Collagen1a mRNA expression was investigated and compared among all groups (Figure 8A-C). ISO increased $\beta$-MHC mRNA expression in MA-ISO compared to age-matched controls ($P < 0.05$) (Fig 8A). Chronic ISO administration did not increase ANF mRNA expression in YA-ISO compared to YA. However, ISO increased ANF mRNA expression in MA-ISO rats compared to MA rats (2670 ± 981 vs. 182 ± 42) ($P < 0.05$) (Fig 8B). ISO induced similar increases in collagen1a mRNA expression in YA-ISO and MA-ISO animals compared to their respective age-matched vehicle controls (Fig 8C).
Discussion

The present results demonstrate that non-senescent, mature adult rats (18 wks) are more susceptible to the deleterious effects of chronic catecholamine excess than younger animals (12 wks). The current findings suggest that abnormal myocardial responses to stress can be detected in animals at an earlier age than has previously been demonstrated (18, 37, 55). Mature adult rats displayed greater reductions in contractile reserve following chronic ISO administration and closely resembled a HF phenotype characterized by a dysregulation in Ca^{2+} handling, a re-engagement of the fetal gene program, and an activation of a key apoptotic enzyme. The current study examined contractile reserve, indexed by changes in +dP/dt_{max}, in response to mechanistically distinct inotropes to reveal systolic impairments in animals that have normal cardiac function at baseline (1, 26, 37). Differences in systolic performance were observed even though milrinone- and digoxin-induced changes in HR and hemodynamics were similar across groups. This observation indicates that the current results are due to innate cardiac factors and are not due to agonist effects on chronotropy, loading conditions, or systemic hemodynamics (55).

The current study was designed to investigate if MA were more sensitive to the effects of chronic catecholamine excess than YA, but the current data do not address whether or not differences in acute cardiovascular responses to ISO exists or develops amongst the ages of animals used in the current study. We did observe similar acute increases in HR in YA and MA animals following acute ISO administration (pilot studies), but it is possible that differing effects of acute ISO on blood pressure and HR may have developed and
contributed to the present results. Potential studies designed to characterize differences in acute cardiovascular responses to ISO over time are severely limited by the fact that ISO induces β-receptor dysfunction, regardless of age (18, 21, 32). In the future, longitudinal studies using radiotelemetry in conscious animals will be needed to systematically determine if acute responsiveness to ISO changes over time and influences subsequent outcomes. Additionally, whether or not the observed changes contribute to decreases in cardiovascular function during post-maturational aging or senescence is beyond the scope of the current study.

In vivo and isolated heart studies comparing mature and senescent animals have established that contractile reserve along with the ability of the myocardium to adapt to added stress diminishes with advancing age (1, 10, 36, 37). ISO has also been demonstrated to cause greater impairments of contractile responses in hearts from rats aged 28-30 months compared to hearts from rats aged 6-8 months (25). Eventhough age-related declines in cardiovascular function are well documented amongst mature adult (8-20wks), old (>80wks), and senescent rodents (>100wks), these differences have not previously been reported to occur earlier in life (15, 19, 21, 23, 34, 46, 48, 53-55).

Because β-receptor dysfunction is known to occur in models of catecholamine excess, the current study utilized clinically relevant inotropic agents that elicit effects independent of direct β-receptor activation (18, 21, 32-34, 41). This strategy minimized the confounding effects of β-receptor desensitization and/or downregulation that likely influenced previous assessments of contractile reserve in this model (19, 33, 34, 39, 41). Milrinone acts distally to the β-receptor, inhibiting the phosphodiesterase (type III) that
specifically degrades cAMP (41). It is unclear if β-receptor dysfunction can alter the availability of cAMP in this model, however many studies have demonstrated efficacy of milrinone in animal and human HF (24, 41). Digoxin has a long history of clinical use for the treatment of congestive heart failure (3, 4, 11, 50). This cardiac glycoside promotes myocardial Ca\(^{2+}\) entry which increases cardiac contractility mainly through the inhibition of the sarcolemmal Na\(^+\), K\(^+\)-ATPase (NKA) (3, 50). There is evidence that inotropic responses to cardiac glycosides can be reduced in models of cardiac hypertrophy and dysfunction. The mechanisms for this effect are controversial but may be explained by underlying Ca\(^{2+}\) handling defects within the cardiomyocytes (3, 4, 11, 27, 50).

There are several potential molecular mechanisms to explain the differing levels of systolic impairment in mature adult and young adult rats following chronic catecholamine excess. Previous reports demonstrating systolic impairments induced by aging or chronic ISO administration implicate factors such as: dysregulations in Ca\(^{2+}\) handling, inductions of the “fetal gene program”, and/or increases in caspase activity (1, 8, 10, 15, 22). Therefore, we conducted an initial survey of these factors in an attempt to identify pathways that may be involved with ISO-induced decreases in contractile reserve.

Disruption in Ca\(^{2+}\) cycling is a critical mechanism underlying impairments in the contractile function of the heart and it is well known that the aging heart is more susceptible to Ca\(^{2+}\) overload (22, 29). In the present experiments, chronic ISO decreased SERCA2a mRNA expression in both YA and MA rats. However, expression in MA-ISO was significantly reduced compared to YA-ISO. These changes would be expected to
decrease the amplitude of Ca\textsuperscript{2+} transients and impair systolic force (22). This data is in agreement with studies demonstrating age-related and ISO-induced decreases in SERCA2a mRNA expression, and with the notion that β-receptor stimulation can reveal underlying impairments in Ca\textsuperscript{2+} handling (1, 2, 15, 40). Present evidence of decreased SERCA2a mRNA expression following chronic ISO administration is supported by functional data showing increases in Tau (Weiss). The current findings are in agreement with studies demonstrating that Tau is prolonged in aging hearts and studies demonstrating that SERCA2a mRNA begins to decline in the rodent at 16 weeks of age (29, 34, 43). There are other potential detriments in Ca\textsuperscript{2+} handling that could explain the observed differences that were not addressed in the current study. The L-type Ca\textsuperscript{2+} channel, the ryanodine receptor, and phosphatase-1 are involved in maintaining normal Ca\textsuperscript{2+} regulation and have been shown to be altered in various models of systolic impairment (37). However, SERCA2a has been identified as an initial Ca\textsuperscript{2+} handling protein affected by aging and the aim of the present study was to evaluate changes expected to occur early in the aging process (1, 23).

Consistent with previous reports, the current study demonstrates significant LV hypertrophy following chronic ISO administration. The expression of β-MHC and ANF, maladaptive hypertrophy markers known to be elevated in models of HF and in humans with heart disease, were increased in MA-ISO compared to YA-ISO rats (13, 53, 17, 52). Similar age-related differences in pathological gene expression have been noted between mature adult and senescent rodents (33, 53, 20). These biochemical differences could explain, in part, the functional cardiac impairments reported in the current study. It is known that small changes in the β-MHC content of the myocardium can induce dramatic
changes in function (33, 52). This gene is reciprocally expressed with the more contractile α-MHC and an increase in β-MHC expression alone has been demonstrated to directly depress cardiac contractile function (20). The current results are consistent with reports that ANF expression increases with the development of cardiac hypertrophy and with reports of ISO-induced increases in ANF expression (13, 17, 38, 39).

ISO-induced hypertrophy and human HF are often accompanied by evidence of apoptosis and increased caspase-3 (36, 38, 47). Caspase-3 activity increased in MA-ISO and was not altered in YA-ISO, supporting the idea that non-senescent animals are more vulnerable to injury and stress than their younger counterparts. This finding is consistent with a previous study showing that caspase-3 overexpression depresses cardiac function in mice, and with a report that caspase-3 expression is elevated in a dog model of HF (14, 45). An in vitro study demonstrated that caspase-3 activation leads to a reduction of contractile reserve in LV cardiomyocytes (30). Additionally, pharmacological inhibition of caspase-3 improved post-ischemic contractile recovery in the isolated rat heart (49). The present in vivo results, along with data from several models of cardiovascular injury, support the notion that caspase activation plays a major role in the development of contractile dysfunction (9, 30, 45, 49). The precise mechanisms of caspase-3-induced reductions in systolic performance are uncertain but may involve perturbations in Ca\(^{2+}\) homeostasis or direct effects on the contractile proteins of the sarcomere (9, 47, 49).

Perspectives and Significance

This study provides, to our knowledge, the first demonstration that non-senescent, adult rats (18 wks) have a greater reduction in contractile reserve following chronic, repeated
bouts of catecholamine excess than younger animals (12 wks). Although several studies report age-related declines in cardiac performance at both the functional and molecular level in senescent animals, the current data suggest that small differences in age can influence cardiovascular adaptations to stress. The present study demonstrates, through the use of mechanistically distinct inotropes, that the detrimental interaction between age and catecholamine excess on the ability to respond to cardiac demand can be detected early in life. Greater impairments in contractile reserve were associated with decreases in SERCA2a expression, increases in β-MHC and ANF expression, and increases in caspase activity. Future investigations are required to further elucidate the interaction between aging and the regulation and relative contribution of these maladaptive processes.
Bibliography


**Figure Legends**

Fig. 1. Baseline contractility of all animals (YA, YA-ISO, MA, and MA-ISO) prior to assessment of contractile reserve. Positive and Negative dP/dtMax were diminished in all groups compared to YA controls. Data are the mean ± SEM (One-way ANOVA followed by a Bonferroni multiple comparisons test; n = 9-14). *P<0.05 vs. vehicle-treated YA rats.

Fig. 2. Baseline values for Tau (Weiss) (A), heart rate (B), and mean arterial pressure (C) in all animals (YA, YA-ISO, MA, and MA-ISO) prior to assessment of contractile reserve. Tau was elevated in YA-ISO and MA-ISO compared to their vehicle-treated controls. There were no differences in HR or MAP across groups. Data are the mean ± SEM (One-way ANOVA followed by a Bonferroni multiple comparisons test; n = 5-9). *P<0.05 vs. age-matched controls

Fig. 3. Effect of Milrinone and Digoxin on +dP/dtMax in YA, YA-ISO, MA, and MA-ISO animals. Infusions of milrinone (20 – 200 μg/kg/min) produced comparable responses in YA-ISO and YA animals (P = 0.052; 2-way repeated measures ANOVA followed by Holm-Sidak post test; n= 4-7) (3A). The response to milrinone (20 – 200 μg/kg/min) was significantly attenuated in MA-ISO compared to MA controls (P =0.001; 2-way repeated measures ANOVA followed by Holm-Sidak post test; n= 4-7) (3B); *P<0.05 vs. vehicle-treated animals at same dose, ‡P<0.05 for effect of treatment (ISO) on the response to serial inotrope infusion.

Infusions of digoxin (20 – 200 μg/kg/min) produced similar responses in YA-ISO and YA animals (P = 0.194; 2-way repeated measures ANOVA followed by Holm-Sidak; n= 4-7) (4A). Contractile response to digoxin (20 – 200 μg/kg/min) was significantly attenuated in MA-ISO compared to MA controls (P = 0.01; 2-way repeated measures ANOVA followed by Holm-Sidak; n= 4-7) (4B). *P<0.05 vs. same dose in vehicle-treated animals, ‡P<0.05 for effect of treatment (ISO) on the response to serial inotrope infusion.

Fig. 4. Effect of chronic ISO administration on cardiac hypertrophy. ISO induced significant increases in LV index (LVI: left ventricular free wall + septum (mg) / tibia length (mm)) in both YA-ISO and MA-ISO rats compared to their age-matched vehicle controls. Additionally, MA-ISO rats demonstrated greater increases in LVI compared to YA-ISO. Data are the mean ± SEM (One-way ANOVA on all groups, followed by a Bonferroni multiple comparisons test; n = 5-9). *P<0.05 vs. age-matched control, †P<0.05 vs. YA-ISO.

Fig. 5. Effect of chronic ISO administration on SERCA2a mRNA expression and caspase-3 activity. ISO induced significant decreases in SERCA2a expression in both YA-ISO and MA-ISO rats compared to their age-matched vehicle controls. Additionally, greater decreases in SERCA2a mRNA expression were detected in MA compared to YA, and in MA-ISO compared to YA-ISO. Data are the mean ± SEM (One-way ANOVA by a Bonferroni multiple comparisons test; n = 4-7). *P<0.05 vs. age-matched control, †P<0.05 vs. YA-ISO, ‡P<0.05 vs. YA.
An increase in caspase-3 activity was detected in MA-ISO animals. Data are the mean ± SEM (One-way ANOVA followed by a Bonferroni multiple comparisons test; n = 4-8). * P<0.05 vs. all groups.

Fig. 6. Effect of chronic ISO administration on hypertrophy-associated gene expression. ISO increased β-MHC mRNA expression in MA-ISO compared to age-matched controls (A); significant increases in ANF mRNA expression were only detected in MA-ISO animals (B); similar increases in collagen1a mRNA expression were detected in YA-ISO and MA-ISO animals. Data are the mean ± SEM (One-way ANOVA followed by a Bonferroni multiple comparisons test; n = 5-9). *P<0.05 vs. age-matched control.
Table 1. Heart rate, systolic arterial pressure, and mean arterial pressure following milrinone or digoxin infusions. Baseline values for HR, SAP, and MAP were not different across all groups (n = 8-14); These values were also not different following the infusions of milrinone (n = 4-7) or digoxin (n = 4-7). Data are the mean ± SEM (one-way ANOVA followed by a Bonferroni multiple comparisons test).

<table>
<thead>
<tr>
<th>Heart Rate (bpm)</th>
<th>BASELINE</th>
<th>MILRINONE (200 μg/kg/min)</th>
<th>DIGOXIN (200 μg/kg/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>YA</td>
<td>318.8 9.3</td>
<td>351.6 14.6</td>
<td>328.7 16.7</td>
</tr>
<tr>
<td>ISO-YA</td>
<td>311 6.5</td>
<td>358.2 8.8</td>
<td>290.3 11</td>
</tr>
<tr>
<td>MA</td>
<td>329.3 8.7</td>
<td>333.7 28.6</td>
<td>311 9</td>
</tr>
<tr>
<td>ISO-MA</td>
<td>308.2 4.6</td>
<td>320.6 15</td>
<td>298 16</td>
</tr>
</tbody>
</table>

| SAP (mmHg)       | YA 123 3.7 | 77.1 3.1                  | 148.8 6.2              |
| ISO-YA           | 112 4.2   | 82.3 4.4                  | 147.7 7.3              |
| MA               | 111 3.9   | 84.8 3.2                  | 133.9 1.2              |
| ISO-MA           | 116.6 5.4 | 81.4 2.2                  | 148.4 4.9              |

| MAP (mmHg)       | YA 97.1 2.7 | 65 3.9                   | 114.8 3               |
| ISO-YA           | 94.9 2.5   | 67.7 3.8                 | 113 5.6               |
| MA               | 92.3 2.5   | 70.5 2.3                 | 107 1                 |
| ISO-MA           | 96.2 3.8   | 68 2.5                   | 110.2 6               |
3A  
**Milrinone Young**

- YA
- YA-ISO

Δ +dP/dt (mmHg × sec⁻¹)

Milrinone (ug/kg/min)

n.s. p = 0.052

3B  
**Milrinone Mature**

- MA
- MA-ISO

Δ +dP/dt (mmHg × sec⁻¹)

Milrinone (ug/kg/min)

† p = 0.001

3C  
**Digoxin Young**

- YA
- YA-ISO

Δ +dP/dt (mmHg × sec⁻¹)

Digoxin (ug/kg/min)

n.s. p = 0.194

3D  
**Digoxin Mature**

- MA
- MA-ISO

Δ +dP/dt (mmHg × sec⁻¹)

Digoxin (ug/kg/min)

† p = 0.01
Quantitative PCR of SERCA2 Expression:

**Top graph:**
- **Y.A.**
- **Y.A.-ISO**
- **MA**
- **MA-ISO**

**Caspase-3 Activity**

**Bottom graph:**
- **Y.A.**
- **Y.A.-ISO**
- **MA**
- **MA-ISO**

Fold increase
A  
\(\beta\)-MHC mRNA expression

![Bar graph showing \(\beta\)-MHC mRNA expression across different groups: YA, YA-ISO, MA, and MA-ISO.

B  
ANF mRNA Expression

![Bar graph showing ANF mRNA expression across different groups: YA, YA-ISO, MA, and MA-ISO.

C  
Collagen1a mRNA expression

![Bar graph showing Collagen1a mRNA expression across different groups: YA, YA-ISO, MA, and MA-ISO.

* denotes statistically significant difference.